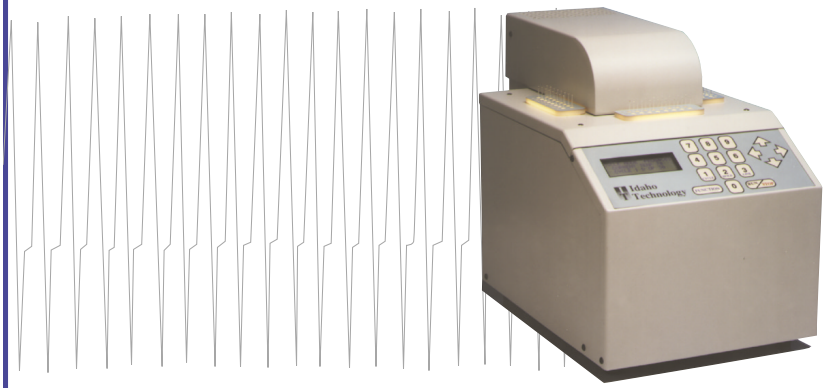
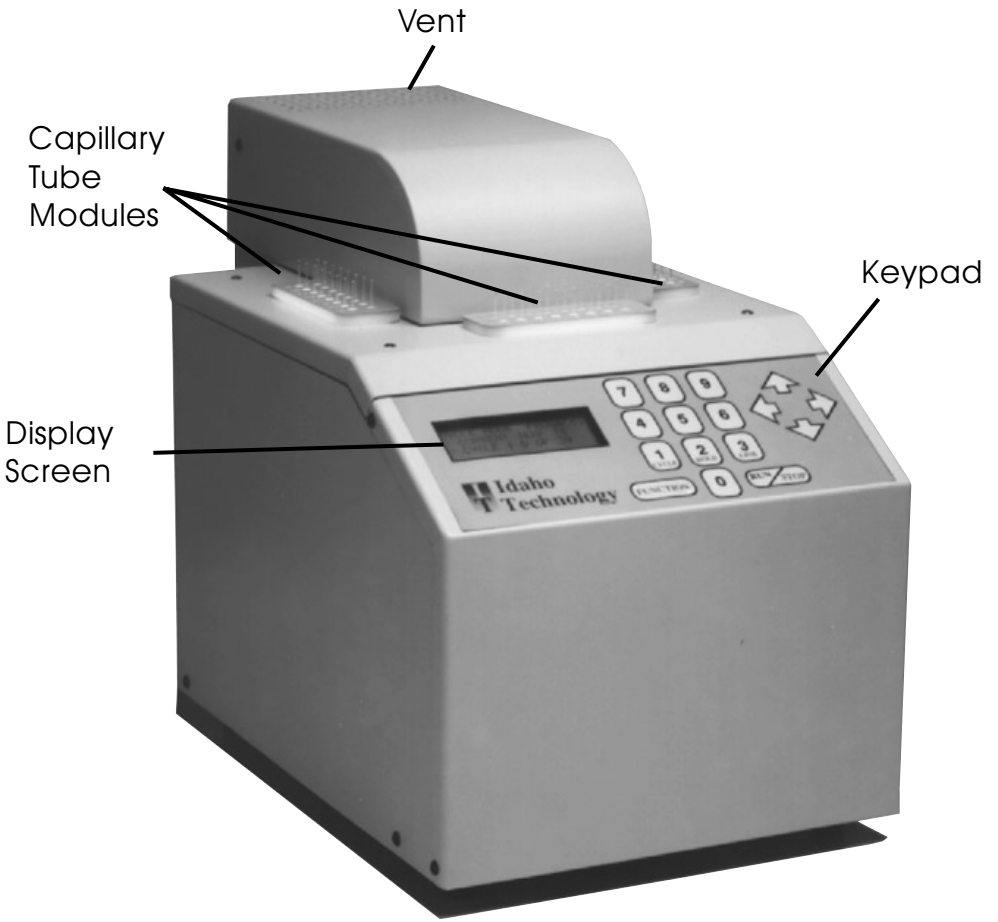
THE RapidCycler®



USER'S GUIDE

Idaho Technology Inc.

The RapidCycler®



Note: This illustration is optimized for printing.

Contents

Introduction 1

Setting Up the RapidCycler 3

Sample Handling 4

Programming 21

Trouble-Shooting 34

Service and Maintenance 38

Warranty and Upgrades 46

Articles 47

Automated polymerase
chain reaction in capillary tubes
with hot air 48

Minimizing the time required
for DNA amplification by efficient heat transfer
to small samples 54

Rapid cycle DNA amplification:
time and temperature optimization 63

RapidCyclist Newsletter 76

Index 76

Introduction

The RapidCycler® is a rapid temperature cycling system based on heat transfer by hot air to samples contained in thin capillary tubes. For most reactions, products can easily be visualized with ethidium bromide on agarose gels after a total reaction time of 15-30 minutes. The rapid temperature response of this instrument can improve product specificity significantly while decreasing the required reaction time by up to an order of magnitude.

Average temperature transition rates in most instruments are commonly about 1° C/sec when metal blocks or water are used for thermal equilibration and samples are contained in plastic microfuge tubes. A significant fraction of the cycle time is spent heating and cooling the sample, as opposed to being spent at optimal temperatures. Long reaction times of 2-6 hours are common, and slow transition rates make it difficult to determine optimal temperatures and times for each stage of the cyclic reactions. Instantaneous temperature changes are not possible because of sample, container and cycler heat capacities. "Second generation" instrumentation can complete 30-cycles in about one hour. It is unlikely any instrument based on samples contained in conical tubes with heating and cooling through a metal block can achieve faster cycles at any price. However, with capillary tubes and air heating, transition rates of 5-10°C/sec are easily obtained. Complete 30-cycle reactions can be finished in as little as 10 min. Biochemical reactions are fast. The RapidCycler is the first instrument engineered to match this speed.

The advantages of an air-cycling system include simplicity, low cost, and rapid temperature cycling. Air is an ideal heat transfer medium which can change temperature quickly because of its low density. Temperature homogeneity problems are solved by rapidly mixing air with a fan to provide homogeneous temperature exposure over the sample containers. The sample container is just as important as the heat transfer medium. An optimal sample container should be water-vapor tight and have:

(i) low thermal mass,
(ii) good thermal conductivity,
(iii) minimal internal condensation,
(iv) easy sample recovery without cross contamination,
(v) No adverse effects on the reaction.

Whatever the container material, temperature equilibration will always be achieved faster if the sample volume is small, if the container wall is thin, and if the surface-to-volume ratio of the sample exposed to the container wall is high. Problems with condensation can be reduced by minimizing the free air space surrounding the sample.

Microfuge tubes are kept water-vapor tight by mechanical closure and, if necessary, overlaid mineral oil. Thermal conductivity is poor because of the material and its thickness (about 1 mm). Internal condensation can occur if mineral oil is not used and particularly if different parts of the tube are at different temperatures. Sample mixing by convection has been used in conical tube instruments. The temperature gradients that cause convection are not a good idea for a temperature-dependent reaction.

Capillary tubes are kept vapor tight by flame closure of the ends. They conduct heat to the sample better than microfuge tubes because of decreased wall thickness (ca. 0.2 mm) and a better surface-to-volume ratio. Dead air space is minimized to prevent significant condensation. Different volumes of a sample can be placed in the same diameter capillary tube so that rapid heat transfer is maintained.

After amplification, the ends of the glass capillaries can be quickly scored with a file and snapped off with less risk of aerosolization and contamination than microcentrifuge tubes. The capillary tubes serve both as a transfer pipette and container for temperature cycling. The reaction product, already containing Ficoll and Sucrose, an electrophoresis indicator dye can be directly emptied into a gel well without exposure to an intermediate pipette tip or to extraction procedures.

Decreasing the heat capacity of the cycling system can markedly decrease the total time required for reactions that require temperature cycling. In addition, air cycling and miniaturization can significantly decrease the costs of reagents and the personnel time required to optimize reactions.

Setting up the RapidCycler

A STORING YOUR RAPIDCYCLER

In choosing a location to set up your RapidCycler, remember that it uses room air for cooling. Keep the RapidCycler open on all four sides to allow air to flow into the air intake beneath the machine. Also, do not set the RapidCycler on any material which may be sucked into or cover the intake.

NOTE: Lab bench paper is particularly effective at blocking the air intake.

Heated air (up to 90°C) is expelled from the top rear of the RapidCycler, so it is important that the exhaust area be kept clear to avoid restricting the airflow through the RapidCycler. **Be especially careful to keep the exhaust area clear of anything that could be damaged by heat especially volatile organic solvents.**

B PROTECTING YOUR RAPIDCYCLER

Plug the power cord into the RapidCycler and into a grounded surge suppressor. The RapidCycler, like all microprocessor controlled equipment, is sensitive to damaging power fluctuations.

Sample Handling

The RapidCycler **is the only instrument** which can approach the kinetic limits of amplification reactions. The high surface area to volume ratio of capillary tubes and the use of air as the cycling medium makes the RapidCycler the fastest thermal cycler in the world. This comes at the cost of non-standard sample handling techniques.

Sometimes it may not be necessary to have the highest possible reaction specificity. In this case it may be more convenient to use thin-walled microcentrifuge tubes. While much slower, these allow the use of standard reagents and protocols. Following are instructions for rapidcycling with (A) GLASS CAPILLARY TUBES and (B) THIN-WALLED MICROCENTRIFUGE TUBES, and (C) CAPILLARY TUBE HANDLING WITH THE RAPIDCYCLER.

A RAPIDCYCLING WITH GLASS CAPILLARY TUBES

Using a capillary based air cycler is different from using a heat block instrument. Samples must be prepared, loaded into the capillary tubes, sealed, and after the reaction is complete, analyzed. Following are instructions for sample preparation, loading and sealing samples into capillary tubes, cycling and after cycling sample handling.

NOTE: Detailed instructions for the preparation of buffers and optimizing protocols can be found in the Rapidcyclist Newsletters section of this manual.

1. SAMPLE PREPARATION

All reactions in glass capillary tubes must contain 250-500 ug/ml bovine serum albumin to prevent surface denaturation of the enzyme. The same high surface-area-to-volume ratio that allows rapid temperature cycling also provides many sites for enzyme inactivation. We recommend three alternatives for adding adequate BSA to your reaction:

1. Add 10X BSA (2.5 mg/ml) to your master mix. You can either make the solution yourself, or it is included in the Optimizer Kit from Idaho Technology.

2. Dilute your concentrated enzyme stock to a 10X enzyme solution with an enzyme diluent containing BSA (10 mM Tris, pH 8.0, 2.5 mg/ml BSA). You can make the diluent yourself or it is included in the Optimizer kit from Idaho Technology.

3. Use one of the 10X buffers included in the Optimizer kit from Idaho Technology that already contains BSA. These buffers are optimized for rapid cycling and include 10, 12, 30, 40 or 50 Mg++ buffers.

If your reaction involves primer extension, briefly denature template nucleic acid before rapid cycling is begun. We recommend linking a preliminary 15-30 sec. hold at 94° C to your rapid cycle program. Prolonged exposure of template to high temperatures is not recommended, especially when long products are desired, because of the possibility of strand breakage. Only partial renaturation occurs on cooling, allowing rapid denaturation to occur during cycling.

If a plasmid is your source of template, cutting the plasmid with a restriction enzyme may increase yield, although it is usually not necessary.

2. PREPARING MULTIPLE SAMPLES

To simplify preparation of multiple samples, we suggest making a master mix containing all solutions common to the samples you intend to run in one well of a microtiter plate with U-shaped wells. If you are not using the buffer/BSA supplied by Idaho Technology, make sure BSA is added to the master mix before the enzyme to avoid possible absorption of enzyme to the surface. Alternately, use enzyme diluent to make a 10X enzyme solution that already contains BSA.

To prepare individual samples, first distribute DNA, primers, or which ever element is unique to each sample into a row of individual wells of the microtiter

plate. Then transfer the appropriate volume of master mix and aspirate several times with the pipette tip to ensure complete mixing. It is helpful to use a set of colored markers to keep track of your samples. A color code can be used both on the rim of the microtiter plate wells and on the upper portion of the capillary tube as colored bands.

Rapid air cycling is optimized for 10 µl samples. Larger samples (25 and 50 µl) can be used but require 10-15 second hold times, which compromise cycling and reaction time.

Larger volumes can be prepared and cycled with no loss of cycling speed by simply loading the solution into several 10 µl capillary tubes at once. As the liquid wicks up into multiple tubes it distributes itself evenly between the tubes.

3. LOADING AND SEALING SAMPLES INTO CAPILLARY TUBES

After the samples have been mixed in individual wells of a microtiter plate, they can be loaded into capillary tubes either singly or using the capillary rack module. To load a single sample, simply insert a capillary tube into the microtiter well containing the sample. Capillary action will draw the sample into the tube. Run the capillary tip along the bottom of the microtiter well to ensure the entire volume is drawn up into the tube.

It is helpful to hold the microtiter plate in one hand and the capillary tube in the other. Tilting the microtiter plate will ensure complete transfer into the capillary tube.

Once loaded, the position of the sample in the tube can be shifted by tilting the tube. Adjust the sample so that it is roughly centered in the capillary tube. Using a lighter, candle, or regular laboratory burner, flame seal **both ends** of the capillary tube. (Fig. 1) Only a few seconds of heating the **extreme tip** of the capillary is necessary. This will take some practice initially, but becomes simple with repetition. With careful observa-

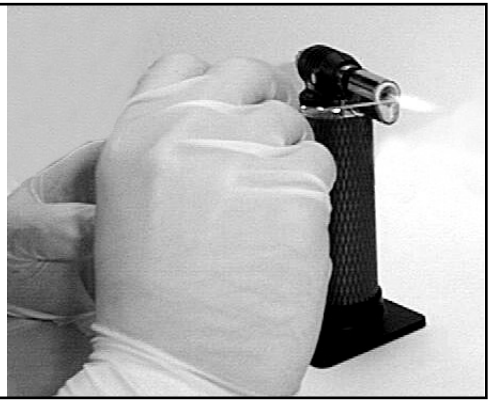


Figure 1.
Seal **extreme tip** of capillary tube in **outer most** portion of the flame. If the tube is heated too much or inserted too far into the flame, it will sag and deform.

tion, the capillary tip appears to "clear up" at the instant of closure. If sealing is not complete, the sample will evaporate during cycling.

After the tubes are sealed, insert them into the holes in the capillary modules located in the instrument top. Push the tubes gently downward until they lightly touch the padded chamber bottom. This will place the sample column completely into the air chamber. Then program and operate the cycler as detailed in section 4, Programming.

Preparing samples using the 16-place capillary tube module (Fig. 2), is similar to preparing tubes individually. However, since the spacing of the holes in the modules are the same as the spacing of microtiter wells, up to sixteen samples can be aspirated simultaneously. The modules also greatly simplify the ordering and labeling of the capillary tubes.

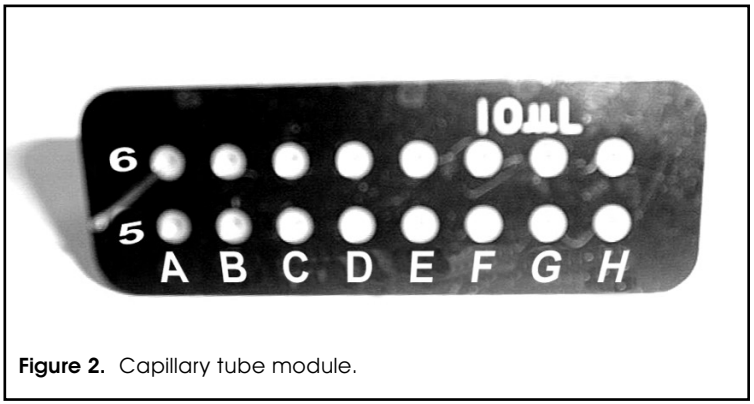


Figure 2. Capillary tube module.

For ease of handling, the modules may be removed from the instrument top. Up to sixteen capillary tubes may be inserted into the modules. After the capillary tubes are in place, align the tubes by pressing against a clean flat surface.

Prepare samples in two rows of a microtiter plate. Then lower the capillary tips into the wells and run them around the bottom of the wells to ensure that all the liquid has been drawn up into the tubes.

Seal the tubes individually, tipping the module to adjust the liquid level. We suggest starting with 8 tubes at a time to gain experience handling and sealing the capillary tubes.

The modules can then be placed back into the machine and cycled. Care must be taken with the capillary tubes when reinserting the module into the machine. Before running, make certain that all three modules are completely seated into the instrument top.

4. Cycling

For information on how to enter and run a program see Section 4, **Programming**. The choice of cycling protocols depends on many factors. Use the tables to adjust temperatures and times when using 25 or 50 µl capillary tubes. Keep in mind that the RapidCycler is optimized for use with 10 µl capillary tubes. The larger 25 or 50 µl tubes require hold times of 10 and 15 seconds at each temperature to allow the sample enough time to come to temperature.

A listing of common cycling protocols for 10, 25, and 50 µl tubes can be found in the Programming section of this manual. See: PREPROGRAMMED RAPIDCYCLER CYCLE PROTOCOLS 51-99, cycles 51-61.

5. After Cycling

After cycling is complete, remove the modules from the instrument and remove tubes. If desired, the modules may be left in place and the tubes removed directly. Verify that no fluid has evaporated from the tubes. If sample has evaporated, the tubes were not completely sealed.

Score the tubes approximately 1 cm from each end using the sapphire cutter supplied. One small stroke with the cutter is sufficient to score the glass. Holding the tube horizontally, gently snap off scored ends.

Next, insert the capillary tube into the white silicone tip of the microaspirator/dispenser approximately 2 cm. While inserting capillary tube into microaspirator/ dispenser you will notice the sample tends to be pushed out of the tube because of back pressure. This requires the user to turn the black knob on the dispenser counterclockwise to prevent the sample from being pushed out of the capillary tube as it is being inserted into the silicon tip.

The user may now dispense the amplified sample from the capillary tube by inserting the capillary tip directly into a gel well and slowly turning the microaspirator/ dispenser knob clockwise to dispense the sample.

NOTE: With extensive use the silicon tip of the microaspirator/dispenser will lose its seat and need to be replaced.

8 Use of Thin Walled Microcentrifuge Tubes with the RapidCycler

1. Introduction

The development of thin walled micro test tubes makes it possible to combine the speed of the air cycling with the convenience of that "universal vessel" of molecular biology, the microcentrifuge tube. While the RapidCycler was developed for use with glass capillaries, it provides excellent results with thin walled microcentrifuge tubes. Using modified sample modules, the RapidCycler can hold up to 48 micro test tubes Figure 1 shows that all 48 positions give a clean, bright, 500 bp product in a DNA amplification from Human genomic DNA.

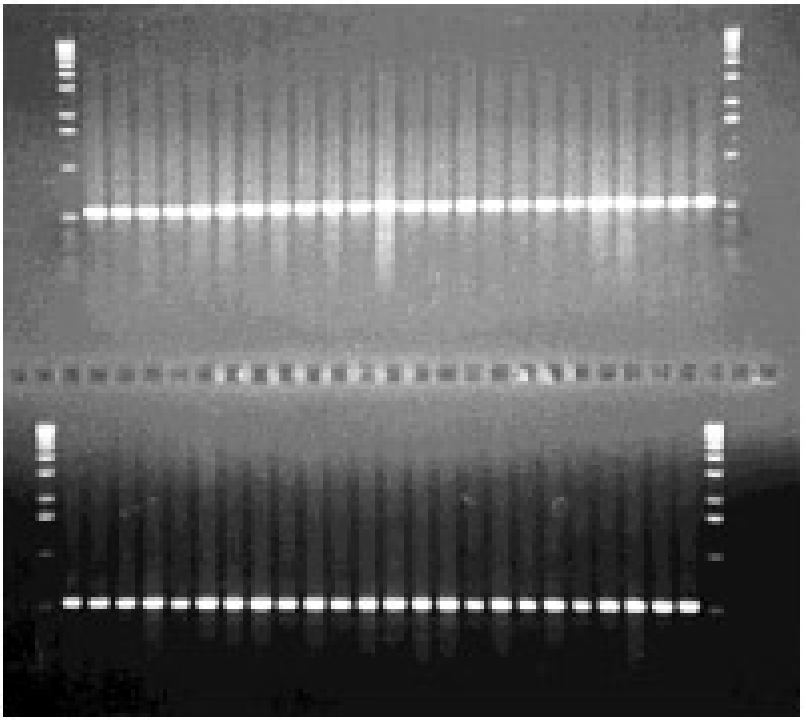


Figure 1. Amplification of a 500 bp target from human genomic DNA in all 48 sample positions of the Air Thermo-Cycler. Reactions volume was 50 μl, no oil overlay. Reactions contained Idaho Technology medium buffer, 200 μM each dNTP, 5 μM each primer (RS/KM), 50 ng human genomic DNA. Cycling parameters were 96° for 30 seconds, then 30 cycles of 96° for 30 seconds, 55° for 30 seconds, 75° for 20 seconds.

Thin walled micro test tubes have many advantages over capillary tubes. First, handling of the sample tube is much simpler; reactions can be made up in the micro test tube, no heat sealing is required, concern about breaking the tubes is eliminated. Second, there is no need to adjust buffers or protocols. The buffers that manufacturers provide with their thermostable polymerases work in these tubes without modification. Published protocols developed in heat block instruments seem to transfer more readily to the RapidCycler when micro test tubes are used.

The thermal properties of thin walled microcentrifuge tubes are much better than their thick walled ancestors, but they are still no match for a capillary tube. Using thin walled microcentrifuge tubes requires a sacrifice in speed and in sample temperature uniformity. A 10 μl reaction that would take 15 minutes in a capillary tube, takes 35 minutes in a thin walled microcentrifuge tube, a 50 μl reaction that would take 20 minutes in a capillary, takes 50 minutes in a microcentrifuge tube.

Because the RapidCycler was developed for capillary tubes the temperature values that you program into the machine, and the temperatures displayed during cycling, reflect what the temperature would be in a 10 μl capillary. When using microcentrifuge tubes you must modify the program parameters to compensate for the thermal differences between capillaries and microcentrifuge tubes.

2. THIN WALLED MICROCENTRIFUGE TUBE CYCLING PROTOCOLS FOR THE RAPIDCYCLER

There are two possible approaches when using microcentrifuge tubes. You can set the machine to the temperature you want, and wait for the microcentrifuge tube to get to that temperature (Figure 2B. This is what the slower heat block cyclers do). This method is slow, but it assures you that no part of your sample is ever over the target temperature. A faster approach is to overheat and under heat the air. This brings the sample to temperature more quickly (Figure 2A. The faster heat block instruments do this), but some parts of your sample may be slightly above or below the target temperatures.

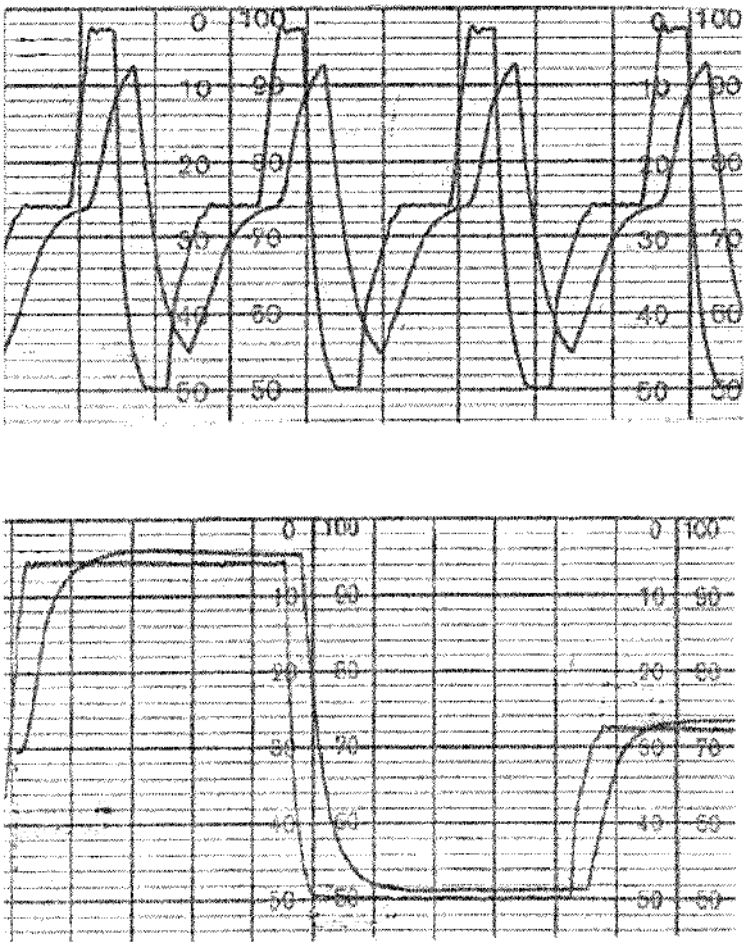


Figure 2. Temperature traces of the hold method (2B) versus the over heat and under heat method (2A). Traces are of air temperature and actual sample temperature. Notice how the sample temperature always lags behind the air temperature, and how the over/under heat method brings the sample to temperature more quickly.

I have had good success with the faster overheat and under heat approach. The following protocols have been successful with a variety of primers and DNA sources and are preprogrammed as cycle number 82-87.

10 µl Reactions

Predenature: 98°C for 10 seconds

Cycle:	Denature	98°C for 10 seconds
	Anneal	40°C to 60°C for 10 seconds (as appropriate for your primers)
	Extend	74°C for 25 nucleotides per seconds

50 µl Reactions

Predenature: 96°C for 30 seconds

Cycle:	Denature	96°C for 30 seconds
	Anneal	40°C to 60°C for 30 seconds (as appropriate for your primers)
	Extend	74°C for 25 nucleotides per seconds

If you prefer the sit and wait approach 10 µl samples require 40 second holds at denaturation and annealing, 50 µl samples 60 second holds at denaturation and annealing. Elongation requires 25 nucleotides per second plus about 15 seconds.

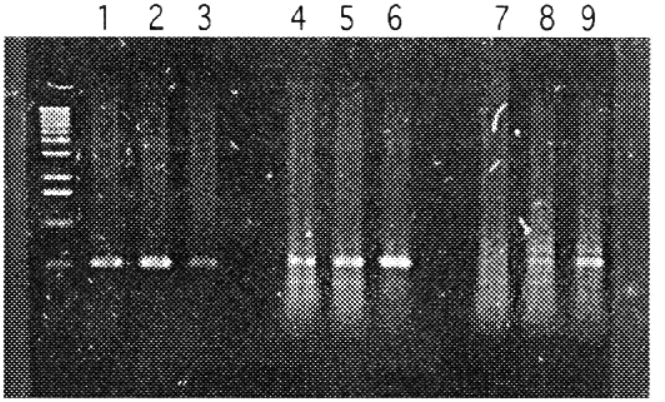


Figure 3. Optimization of RS/KM primer pair in microfuge tubes. Lanes 1-3: 60°C annealing, 4, 3 and 2 mM MgCl. Lanes 4-6: 50°C annealing, 4, 3 and 2 mM MgCl. Lanes 7-9: 40°C annealing, 4,3 and 2 mM MgCl. 10 µl reaction volume, no oil, 10 sec. holds at annealing and denaturation.

3. OPTIMIZATION OF REACTIONS IN THIN WALLED MICROCENTRIFUGE TUBES

The same optimization protocol that has been recommended in capillaries (Optimizing Rapid Cycle DNA Amplification Reactions, Rasmussen and Reed, Rapid Cyclist 1:1-5 (1992) has provided excellent results in thin walled microcentrifuge tubes.

Optimal reaction conditions are found by running amplifications at 40C°, 50C° and 60C° with 2 mM, 3 mM and 4 mM MgCl at each temperature. This allows you to test 9 different stringencies, while only requiring you make up three different reaction mixes.

I have used this optimization protocol successfully with Idaho Technology buffers (low, medium and high MgCl buffers), Promega 10X Taq buffer and Stratagene 10X Pfu buffer.

4. ARE MINERAL OIL OVERLAYS REQUIRED?

The thin walled microcentrifuge tube holders for the RapidCycler put the entire tube inside the reaction chamber. This keeps the whole tube at the same temperature and thus reduces condensation. A small amount of condensation occurs on the leeward side of the tubes, but I have not found this to be a practical problem, even for 10 μl reactions. While a little mineral oil does stop this condensation, in general, oil is not needed for 10 μl reactions.

50 μl reactions show minimal condensation, but will occasionally pop open during reactions if no oil is used. The frequency with which this occurs seems to vary with reaction buffer and with tube manufacturer, so you may wish to experiment with your particular combination.

5. REAL VERSUS SET TEMPERATURES

The fast protocols given above both give a sample temperature of 94° denaturation and 72° to 74° extension. The actual sample annealing temperature may not be important to you if you optimize the reaction experimentally as recommended above. If you do need a particular annealing temperature, the value you should set can be calculated using the equations in figure 4. I have provided graphs for 10 μl reactions with a 10 second hold (figure 4A) and for 50 μl reactions with a 30 second hold (figure (4B).

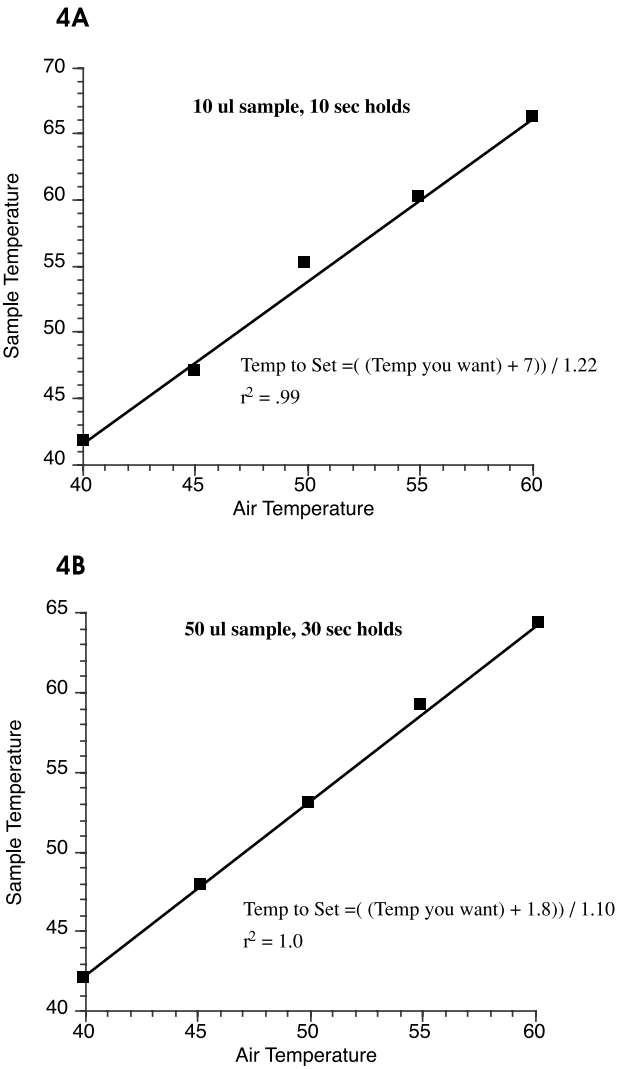

4A
10 ul sample, 10 sec holds
Sample Temperature
Air Temperature
Temp to Set =((Temp you want) + 7)) / 1.22
r2 = .99
4B
50 ul sample, 30 sec holds
Sample Temperature
Air Temperature
Temp to Set =((Temp you want) + 1.8)) / 1.10
r2 = 1.0

Figure 4. Linear relationship between the temperature programmed into the air cycler and the actual sample temperature for thin walled capillary tubes. 4A: 10 µl samples, 10 second holds, no oil overlay. 4B: 50 µl samples, 30 second holds, no oil overlay.

CAPILLARY TUBE HANDLING with the RapidCycler

One of the biggest concerns for new users of air-cyclers is the handling and sealing of glass capillary tubes. While they are a bit more difficult to use than the traditional microcentrifuge tube, the rapid cycle times and temperature homogeneity made possible by the capillaries makes them more than worth the extra trouble. After a little practice, you may wonder why you ever worried.

1. SINGLE TUBE HANDLING

Mixing the Sample

You can mix your reaction in any sort of container, I use low protein absorbing microtiter dishes (IT#2590). Take care at the mixing step as one of the most common causes of reaction failure is forgetting a component of the reaction (see "The 10 most common mistakes", Rapid Cyclist 2:11-12). The chances of leaving something out can be reduced by making up "master mixes" that contain everything but primer and template. The mix can be stored at 4° C for up to 3 months (see "Reaction mixes and buffer recipes", Rapid Cyclist 2:9).

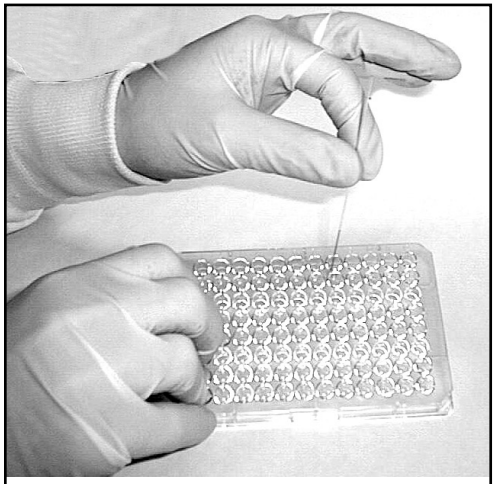


Figure 1. Tipping the capillary tube sideways to increase the rate of liquid uptake.

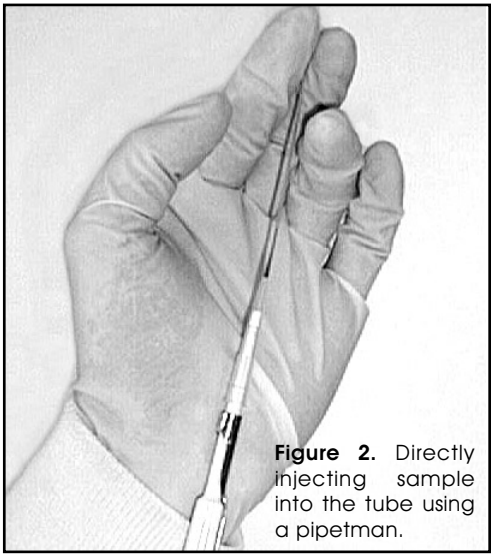


Figure 2. Directly injecting sample into the tube using a pipetman.

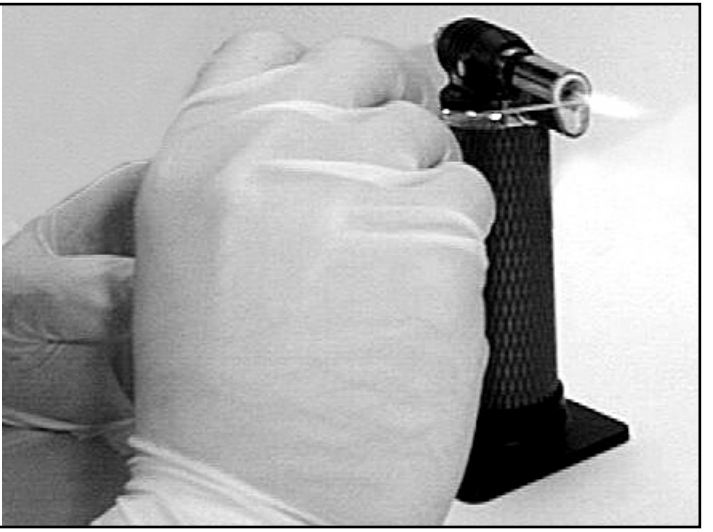


Figure 3. Sealing capillary with a Blazer mini butane torch.

Loading the capillary

Glass capillary tubes are easily loaded by capillary action. You can increase the rate of liquid uptake by tipping the capillary tube sideways (Figure 1). You can also load the capillaries using a Drummond microaspirator (IT#1690) to draw the reaction mix up into the tube, or you can use a pipetman to directly inject sample into the tube (Figure 2).

The 10 µl size tubes hold 2.2 µl/cm and can be used for reaction volumes from 5 to 15 µl. The 10 µl capillaries come to temperature so quickly that they require no holds at denaturation or annealing. The 50 µl tubes hold 9 µl/cm and are useful for reaction volumes from 15 to 70 µl. These tubes require a 15 second hold at the denaturation and annealing temperature.

Sealing the capillary

The glass capillaries sold by Idaho Technology are made out of a high sodium, low melting temperature glass. This makes them very easy to flame seal with just about any flame. They can be sealed with a Bic lighter, a Bunsen burner, a candle, or a Blazer mini propane torch (Figure 3, IT#2721).

After the capillary is loaded, tip the tube to center the liquid. Hold the tube in the center and place the end just into the flame. Rotate the tube in the flame by rolling it between your thumb and index finger. You should be able to see the glass slowly close in on itself. Try to avoid leaving the tube in the flame too long, as you can end up with a big glob of glass which will not fit into the holder . This is more likely in very hot flames. Cutting down the air to the flame will cool these burners down and make the capillaries easier to seal.

You can confirm that the end is sealed by looking carefully at the end for a continuous wall of glass around the end. You can also confirm sealing by blowing on the hot end of the capillary and watching to see if the liquid moves toward the end of the capillary as the glass cools (This is more dramatic for the first seal than the second).

Repeat the sealing process on the other end and then insert the tube into the capillary holding module. A module rack (IT#1735) makes these manipulations easier.

Figure 4. Scoring capillary ends with sapphire cutter.

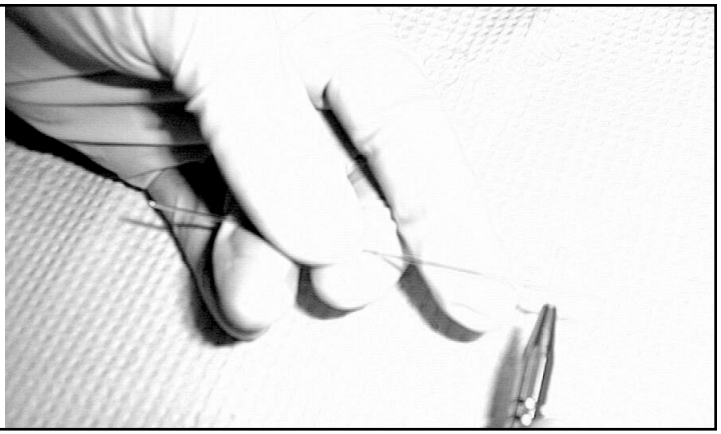
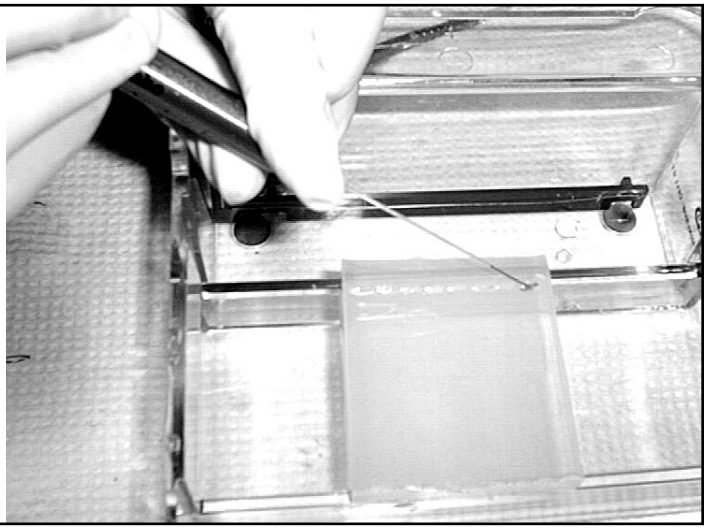


Figure 5. Using capillary tube as a "pipet tip" and directly loading sample into gel.



Sample Recovery

After your reaction is done you pull the tube from the module, lightly score the two ends with a sapphire cutter (Figure 4, IT#1691) and break off the ends. The capillary tube then becomes a "pipet tip" for the Drummond microaspirator (IT#1690) and can be used to directly load your sample into a gel (Figure 5), or into a storage tube.

Beware, the pressure caused by sliding the capillary into the microaspirator can cause your sample to be blown out of the tube. This is easily prevented by dialing the microaspirator back a bit as you insert the capillary tube. The silicon tips of the microaspirator wear out quite quickly, so if your microaspirator stops working try replacing the tip (IT#1870).

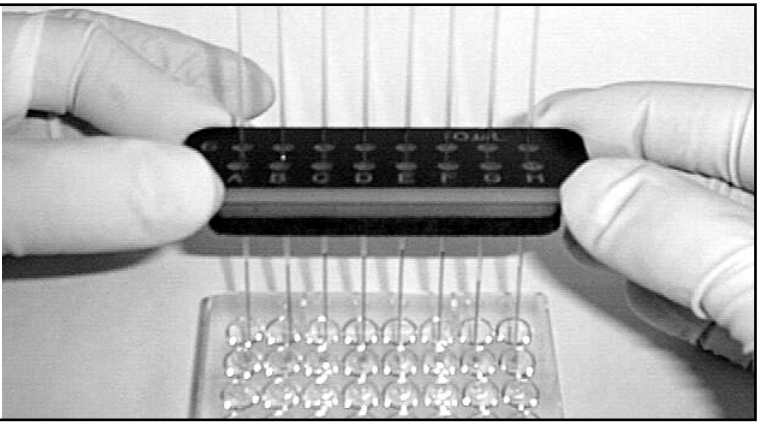
2. MULTIPLE TUBE HANDLING

Once you get single sample handling down, you may want to try some of these "advanced" multiple sample handling tricks.

Eight Sample Handling

When sample modules are made with microtiter spacing it is possible to mix up eight samples at a time in a microtiter dish and draw them up simultaneously by capillary action (Figure 6). All eight samples can be centered by tilting the module and then the tubes can be sealed by passing the tubes through a flame one at a time (Figure 7). Once the reaction is done you can score all eight tubes at once by lightly drawing the sapphire knife across the top of the module (Figure 8) and then breaking off each tube top (Figure 9). Press the module down to the other end of the capillary tubes and repeat the scoring and breaking.

Figure 6. Mixing eight samples at a time and drawing them up simultaneously with capillary action.



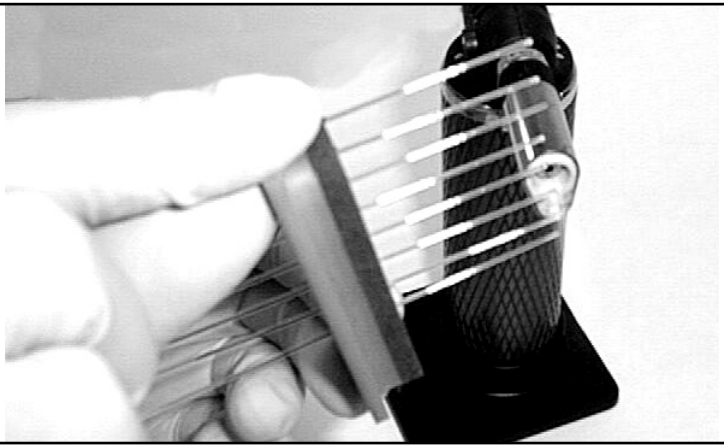


Figure 7. Sealing capillaries by passing the tubes through the flame one at a time.

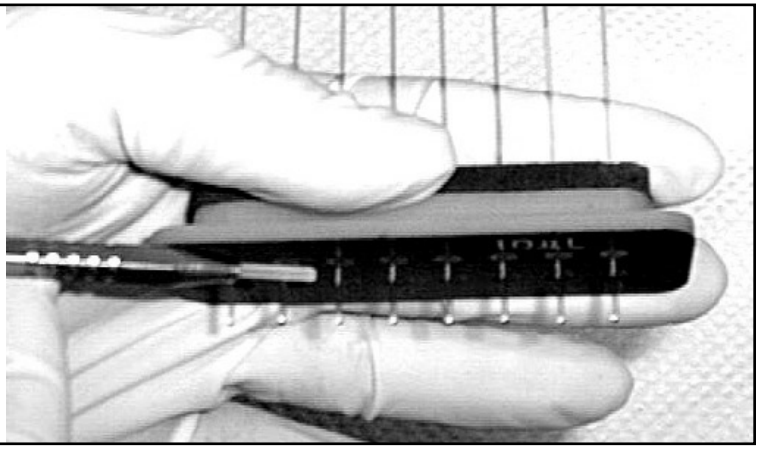


Figure 8. Scoring all eight tubes at once by lightly drawing the sapphire knife across the top of the module.

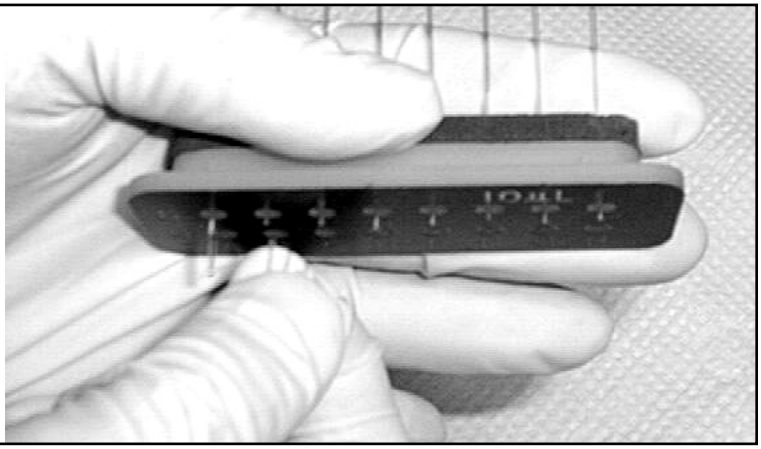


Figure 9. Breaking off tube top after scoring.

Sixteen Sample Handling

After mastering the eight sample tricks, you may want to try 16 at a time. All sixteen tubes in the module can be filled simultaneously by capillary action, similar to the process for sampling 8 tubes. After centering the samples the two rows of eight tubes can then be staggered off from each other by pressing the tubes down on a bench top. The bottom of the first row of eight tubes, and the top row of the second row of eight can then be sealed one at a time by passing through the flame. The staggered rows can then be switched and the remaining two ends can be sealed. After the reaction is done the ends can be scored as done in the eight sample example.

Programming

A OVERVIEW: PROGRAM MODES AND USER INTERFACE

1. RAPIDCYCLER FUNCTION:

When you switch the RapidCycler on, a title screen which contains the software version number is displayed while the controller boots up. After a few seconds, the RAPIDCYCLER FUNCTION screen appears. This is the main menu of the RapidCycler. To enter one of the three operating modes of the RapidCycler simply press the corresponding number:

```
RAPIDCYCLER FUNCTION:
 1. CYCLE MODE
 2. HOLD MODE
 3. LINK MODE
```

To return to the RAPIDCYCLER FUNCTION screen from within the three modes, press the FUNCTION key. If you have pressed RUN/STOP in any of the modes while you are running a program, you must first stop the program by pressing RUN/STOP, then press FUNCTION to return to the RAPIDCYCLER FUNCTION screen.

At start-up, program number 01 in each of the three modes is reset to the standard parameters found in the Program Tables at the end of this section. Each of the other 98 programs in each mode retain whatever values have been entered in them.

If you have not already done so, you may wish to familiarize yourself with the function of the machine by entering each of the three modes and pressing RUN/STOP. Cycle program C-01 will take about 15 minutes but can be stopped at any time by pressing RUN/STOP. Hold program H-01 only takes a few minutes. Link program 01 runs hold program H-01 and cycle program C-01 in sequence and can also be interrupted at any time by pressing RUN/STOP.

2. USER INTERFACE: EDITING NUMBERS

Programming the RapidCycler is easy. It is done by first entering Cycle, Hold, or Link mode. Move the blinking cursor using the cursor keys (arrows), and enter the desired values using the numeric keypad.

When editing a number, remember to type in the same number of digits as the number you are editing. For example, if you want to change the program number from "01" to "02", type "0" and "2" - rather than just typing "2". Programs 02 through 99 in each mode will be remembered by the RapidCycler even if the machine is turned off.

Note that there are not any numbers to edit when the RapidCycler is in the RAPIDCYCLER FUNCTION mode or while running a program, therefore no blinking cursor will be displayed.

B CYCLE MODE

When you enter CYCLE MODE the screen should appear as follows:

```
CYCLE MODE  PROG#C01
TEMP D94   A55   E72
TIME 0:00 0:00 00:15
SLOPE=9.9 CYCLES=30
```

1. DESCRIPTION OF CYCLE MODE PARAMETERS

PROG# C01		Program number C (01 THROUGH 99)
TEMP		Temperatures in °C (~30°C - 99°C)
	D	Typically 90° - 96°
	A	Typ. 40° - 68°
	E	Typ. 70° - 74°
TIME		Corresponding hold times in minutes and seconds
	D	0 sec for 10µl sample in capillary tube
	A	0 sec for 10µl sample in capillary tube and highest stringency
	E	1 sec/50 bp for products < 500 bp. 1 sec/25 bp for products < 2 kbp 1 sec/15 bp for products < 5 kbp
SLOPE		Ramp rate between A and E in °C/sec. Typically 9.9 for highest stringency 2 - 6 for low stringency.
CYCLES		Number of cycles

2. CHANGING BETWEEN AND EDITING PROGRAMS

Change to program #C03 by pressing 0 and 3. The parameters should all be null. Use the cursor keys and numeric keys to enter the following temperatures, hold times, slope and cycle count.

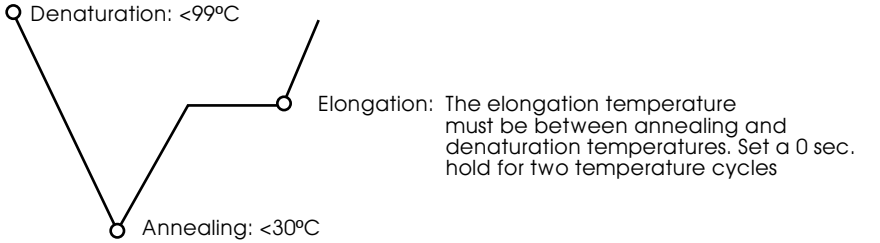
```
CYCLE MODE   PROG#C03
TEMP D94   A65   E72
TIME 0:00 0:00 00:15
SLOPE=9.9    CYCLES=3
```

3. A CYCLE PROGRAM MUST MEET THE FOLLOWING CRITERIA TO RUN

D must be greater than E
E must be greater than or equal to A
For two temperature cycling, set A=E with a 0 second hold at A.
Slope must be greater than 0
Cycles must be greater than 0

Since there are many programs available it may be difficult to remember which programs "belong" to whom. We suggest making copies of the Program Tables at the end of this chapter and posting them near the RapidCycler. Name the Cycle, Hold, and Link programs and thereby "claim" them as your own.

Diagram showing restraints when programming temperature parameters.


Denaturation: <99ºC
Elongation: The elongation temperature must be between annealing and denaturation temperatures. Set a 0 sec. hold for two temperature cycles
Annealing: <30ºC

To edit a program, tab to the PROG# position and enter both digits of the program you wish to modify. The program you were in will be saved automatically as you exit to the new program number. Keep in mind that whenever you enter numbers, the program is changed. If you edit a program that someone else routinely uses in a link program they may not see the changes you have made and it may ruin their run.

4. RUNNING A CYCLE PROGRAM

After all required parameters are entered, you are ready to run the program. Press the "RUN/STOP" key to start the program. Should you want to stop the program before it reaches completion, press the "RUN/STOP" to halt the program. The RapidCycler will then return to the CYCLE MODE screen.

If you try to run a program that does not meet the criteria for a valid cycle program, the RapidCycler will display an error message and beep, then return to the program you tried to run. See the "Changing between and editing programs:" section earlier in this chapter for a list of the cycling criteria.

When running a cycle program the RapidCycler displays the current temperature as well as the cycle count. Line 3 of the display: "CYCLE 1 D OF 30" describes the cycling status as being on cycle #1 of 30 cycles.

After completion of a program, the RapidCycler will display "PROGRAM COMPLETED" and prompt you to "PRESS ANY KEY" to continue. While waiting for you to press a key, the RapidCycler will beep every thirty seconds to remind you that it is finished. Once a key is pressed, the RapidCycler will return to the CYCLE MODE screen for the program you have just completed.

C HOLD MODE

When you enter HOLD MODE the screen should appear as follows.

```
HOLD MODE     PROG#H01
TEMPERATURE=94
TIME=00HR 00MN 15SEC
```

Description of HOLD MODE parameters:

PROG# H	Program number H(01 THROUGH 99)
TEMPERATURE	Temperature in °C (~30°C - 99°C)
TIME	Hold time in hours, minutes, and seconds

1. CHANGING BETWEEN AND EDITING PROGRAMS

Change to program #H03 by pressing 0 and 3. The parameters should all be null. Use the cursor keys and numeric keys to enter a temperature of 94° and hold time of 15 seconds. The screen should appear as follows:

```
HOLD MODE     PROG#H03
TEMPERATURE=94
TIME=00HR  00MN 15SEC
```

Now create another hold program for an extended elongation period after cycling. Select program 04 and enter 72° for 15 seconds.

```
HOLD MODE     PROG#H04
TEMPERATURE=72
TIME=00HR  00MN 15SEC
```

The only requirement for a hold program to run is that the temperature can not be 0 or greater than 99, however, if a temperature below room temperature is entered the machine will be unable to reach it.

2. RUNNING A HOLD PROGRAM

Press the "RUN/STOP" key to start the program. If you wish you may also press the "RUN/STOP" to halt the program.

While running a HOLD MODE program the RapidCycler displays the current temperature and begins a count-down once the desired temperature has been reached.

After completion of a program, the RapidCycler will display "PROGRAM COMPLETED" and prompt you to "PRESS ANY KEY" to continue. While waiting for you to press a key, the RapidCycler will beep every thirty seconds to remind you that it is finished. Once a key is pressed, the

RapidCycler will return to the HOLD MODE screen for the program you have just completed.

D LINK MODE

When you enter LINK MODE the screen should appear as follows.

```
LINK MODE        PROG#01
H01-C01-XXX-XXX-XXX-
XXX-XXX-XXX-XXX-XXX
```

Description of LINK MODE parameters:

PROG#	Program number (01 through 99)
10 LINK SEGMENTS	Up to 10 cycle or hold programs to be run sequentially. Cycle programs are designated CXX and hold programs are HXX.

1. CHANGING BETWEEN AND EDITING PROGRAMS

Link mode can be programmed to run up to ten cycle or hold programs sequentially, but the programs to be linked must fulfill all the criteria for cycle or hold programs for the link program to run to completion. Segments are run in order from the upper left to lower right. Empty segments are skipped.

Change to program #03 by pressing 0 and 3. The parameters should all be null, or 0. Press the right arrow cursor key to enter the first link segment. To link hold or cycle programs using a link program, you must enter 3 digits for each segment. The first digit must be either a "1" or "2", which represent a "C" for Cycle program or "H" for Hold program respectively. Note that the "1" key has the word "CYCLE" on it and the "2" key has the word "HOLD" on it. The next two digits represent the program number of that cycle or hold program.

For example, if you want to link an extended denaturation to a cycle program then finish with an extended elongation, enter hold program #03, cycle program #03, and hold program #04 in sequence. Tab the cursor to the first segment (right arrow cursor key) and type "203" for hold program #03. Tab to the next segment and enter "103" for cycle program #03. Finally, tab to the third segment and enter "204". Note that when you tab away from a link segment, the first digit is changed from a "2" to an "H" or from a "1" to a "C".

If you have not set up cycle program C03 and hold programs H03 and H04 as described in the CYCLE MODE and HOLD MODE sections of this chapter, you may wish to modify them now. Otherwise the link program will not run properly.

2. RUNNING A LINK PROGRAM

Press the "RUN/STOP" key to start the program. If you wish you may press the "RUN/STOP" to halt the program and the RapidCycler will return to the LINK MODE screen.

Running a link program displays the appropriate screen (cycle or hold) for each individual link segment and adds a fourth line to the display of each screen. This additional line lets you know what link segment you are on.

Should you try to run a program that is not valid, the RapidCycler will display an error message, beep, and return to the failed program.

E RAPIDCYCLER'S MEMORY

1. FACTORY-SET PROGRAMS

The RapidCycler is preprogrammed with 32 cycle programs, 51 hold programs, and 10 link programs. For a description of these programs, see the accompanying pages. These programs, in addition to any you may add, will remain in memory even when the RapidCycler is turned off. However, this means that once a preprogrammed protocol is altered, the original program is lost and the altered program will remain in memory. There is one exception, however. In each mode, program #1 is reset to default values whenever the RapidCycler is powered up. This means that modifications to program #1 in cycle hold and link modes will not be saved when the RapidCycler is turned off.

2. PROGRAM TABLES

The tables on the following pages list the preprogrammed cycle, hold, and link programs. Each table also includes space for you to add your own programs. We recommend making copies of these tables and posting them near the RapidCycler.

PREPROGRAMMED RAPIDCYCLER HOLD PROGRAMS

	Temp.	Hrs.	Min.	Sec.			Temp.	Hrs.	Min.	Sec.
H01	94	0	0	15		H51	99	0	0	15
H02	72	0	0	30		H52	96	0	1	0
H03						H53		0	0	30
H04						H54		0	0	15
H05						H55	94	0	1	0
H06						H56		0	0	30
H07						H57		0	0	15
H08						H58	92	0	1	0
H09						H59		0	0	30
H10						H60		0	0	15
H11						H61	90	0	0	15
H12						H62	74	0	10	0
H13						H63		0	2	0
H14						H64		0	0	30
H15						H65	72	1	0	0
H16						H66		0	4	0
H17						H67		0	1	0
H18						H68		0	0	30
H19						H69		0	0	15
H20						H70		0	0	7
H21						H71	60	0	1	0
H22						H72		0	0	30
H23						H73		0	0	15
H24						H74		0	0	7
H25						H75	55	0	1	0
H26						H76		0	0	30
H27						H77		0	0	15
H28						H78		0	0	7
H29						H79	50	0	1	0
H30						H80		0	0	30
H31						H81		0	0	15
H32						H82		0	0	7
H33						H83	45	0	1	0
H34						H84		0	0	30
H35						H85		0	0	15
H36						H86		0	0	7
H37						H87	42	0	1	0
H38						H88		0	0	30
H39						H89		0	0	15
H40						H90		0	0	7
H41						H91	40	0	1	0
H42						H92		0	0	30
H43						H93		0	0	15
H44						H94		0	0	7
H45						H95	35	0	1	0
H46						H96		0	0	30
H47						H97		0	0	15
H48						H98		0	0	7
H49						H99	30	0	1	0
H50										

PREPROGRAMMED RAPIDCYCLER CYCLE PROTOCOLS 1-50

		D	A	E	Cycles	Slope			D	A	E	Cycles	Slope
C01	Temp	94	55	72	30	2.0	C26	Temp					
	Time	0	0	15'				Time					
C02	Temp	94	45	72	30	2.0	C27	Temp					
	Time	0	0	15'				Time					
C03	Temp						C28	Temp					
	Time							Time					
C04	Temp						C29	Temp					
	Time							Time					
C05	Temp						C30	Temp					
	Time							Time					
C06	Temp						C31	Temp					
	Time							Time					
C07	Temp						C32	Temp					
	Time							Time					
C08	Temp						C33	Temp					
	Time							Time					
C09	Temp						C34	Temp					
	Time							Time					
C10	Temp						C35	Temp					
	Time							Time					
C11	Temp						C36	Temp					
	Time							Time					
C12	Temp						C37	Temp					
	Time							Time					
C13	Temp						C38	Temp					
	Time							Time					
C14	Temp						C39	Temp					
	Time							Time					
C15	Temp						C40	Temp					
	Time							Time					
C16	Temp						C41	Temp					
	Time							Time					
C17	Temp						C42	Temp					
	Time							Time					
C18	Temp						C43	Temp					
	Time							Time					
C19	Temp						C44	Temp					
	Time							Time					
C20	Temp						C45	Temp					
	Time							Time					
C21	Temp						C46	Temp					
	Time							Time					
C22	Temp						C47	Temp					
	Time							Time					
C23	Temp						C48	Temp					
	Time							Time					
C24	Temp						C49	Temp					
	Time							Time					
C25	Temp						C50	Temp					
	Time							Time					

PREPROGRAMMED RAPIDCYCLER CYCLE PROTOCOLS 51-99

10µl reactions up to 500 bp. product length

		D	A	E	Cycles	Slope
C51	Temp	94	65	72	30	2.0
	Time	0	0	15		
C52	Temp	94	60	72	30	2.0
	Time	0	0	15		
C53	Temp	94	55	72	30	2.0
	Time	0	0	15		
C54	Temp	94	50	72	30	2.0
	Time	0	0	15		
C55	Temp	94	45	72	25	2.0
	Time	0	7'	10'		

25µl reactions up to 1000bp. product length

		D	A	E	Cycles	Slope
C56	Temp	94	65	72	30	2.0
	Time	10'	10'	35		
C57	Temp	94	55	72	30	2.0
	Time	10'	10'	35		
C58	Temp	94	45	72	30	2.0
	Time	10'	10'	35		

50µl reactions up to 1000bp. product length

		D	A	E	Cycles	Slope
C59	Temp	94	65	72	30	2.0
	Time	15	15	35		
C60	Temp	94	55	72	30	2.0
	Time	15	15	35		
C61	Temp	94	45	72	30	2.0
	Time	15	15	35		

Low stringency reactions ie. RAPD, Diff. Disp.

		D	A	E	Cycles	Slope
C63	Temp	92	42	72	2	1.0
	Time	30"	7'	1'10"		
C64	Temp	92	42	72	2	1.0
	Time	0	7'	1'10"		
C65	Temp	94	40	72	2	1.0
	Time	0	7'	5'		
C66	Temp	94	42	72	5	1.0
	Time	0	7'	5'		

Touchdown

		D	A	E	Cycles	Slope
C67	Temp	94	55	72	5	2.0
	Time	0	0	15		
C68	Temp	94	54	72	1	2.0
	Time	0	0	15		
C69	Temp	94	53	72	1	2.0
	Time	0	0	15		
C70	Temp	94	52	72	1	2.0
	Time	0	0	15		
C71	Temp	94	51	72	1	2.0
	Time	0	0	15		
C72	Temp	94	50	72	21	2.0
	Time	0	0	15		

Test Programs

		D	A	E	Cycles	Slope
C73	Temp	94	65	72	3	2.0
	Time	0	0	10'		
C74	Temp	94	55	72	3	8.0
	Time	0	0	10'		
C75	Temp	98	40	72	3	6.0
	Time	0	7'	10'		
C76	Temp	98	45	72	3	4.0
	Time	30'	30'	30'		
C77	Temp	94	55	72	3	2.0
	Time	30'	30'	30'		
C78	Temp	90	65	72	3	1.0
	Time	30'	30'	30'		

Bum In

		D	A	E	Cycles	Slope
C79	Temp	94	45	72	99	9.9
	Time	15'	15'	10'		
C80	Temp	94	65	72	99	5.0
	Time	0	0	0		
C81	Temp	94	45	72	99	1.0
	Time	30'	30'	10'		

10µl Reactions in Thin Walled Tubes

		D	A	E	Cycles	Slope
C82	Temp	98	60	72	30	2.0
	Time	10'	10'	35		
C83	Temp	98	50	72	30	2.0
	Time	10'	10'	35		
C84	Temp	98	40	72	30	2.0
	Time	10'	10'	35		

50µl Reactions in Thin Walled Tubes

		D	A	E	Cycles	Slope
C85	Temp	96	60	72	30	2.0
	Time	30'	30'	35		
C86	Temp	96	50	72	30	2.0
	Time	30'	30'	35		
C87	Temp	96	40	72	30	2.0
	Time	30'	30'	35		

PREPROGRAMMED RAPIDCYCLER CYCLE LINK PROTOCOLS 1-50

	1	2	3	4	5	6	7	8	9	10	Description:
L01	H01	C01									15s@94-Standard Cycle
L02	H01	C01	H02								15s@94-Std. Cycle-30s@72
L03											
L04											
L05											
L06											
L07											
L08											
L09											
L10											
L11											
L12											
L13											
L14											
L15											
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L42											
L43											
L44											
L45											
L46											
L47											
L48											
L49											
L50											

PREPROGRAMMED RAPIDCYCLER CYCLE LINK PROTOCOLS 51-99

	1	2	3	4	5	6	7	8	9	10	Description:
L51	H01	C01									Hard denature-Standard Cycle
L52	H01	C01	H02								15s@94-Std. Cycle-30s@72
L53	C63	C64	H66								RAPD
L54	C65	C66	C55								Degenerate Primer
L55	H01	C67	C68	C69	C70	C71	C72	H02			Touchdown 50-55∞ Annealing
L56											
L57											
L58											
L59											
L60											
L61											
L62											
L63											
L64											
L65											
L66											
L67											
L68											
L69											
L70											
L71											
L72											
L73											
L74											
L75	H57	H77	H69	H51	H85						TEST-Calibration
L76	C73	C74	C75	C76	C77	C78	H56	H84	H64	H99	TEST-Strip Chart
L77	C79	C80	C81								TEST-Burn In
L78											
L79											
L80											
L81											
L82											
L83											
L84											
L85											
L86											
L87											
L88											
L89											
L90											
L91											
L92											
L93											
L94											
L95											
L96											
L97											
L98											
L99											

Trouble-Shooting

Phone numbers to call for service problems:
US and Canada: 1-800-735-6544
Outside the US: 1 (801) 736-6354
Fax: 1 (801) 588-0507

E-mail addresses:
Idaho Technology: it@idahotech.com
User's Group: lightcycler@idahotech.com

Web address:
www.idahotech.com

Q. There is no display when the instrument is turned on?

A. Make sure that it is plugged in, if so then check electrical fuses as per instructions found in the Service and Maintenance section of this manual. If the problem persists, please call our service department at the appropriate number listed at the beginning of this section.

Q. When RUN is pressed nothing happens?

A. Be sure the proper protocol screen is visible on the LCD display as per the instructions found in the Programming section of this manual. If the desired protocol is being displayed, press the RUN/STOP button several times and watch the display. The display should change with each press of the RUN/STOP button, shifting from a "SET PROTOCOL" screen to a "RUN" screen as shown in the Programming section of this manual. When the "RUN" screen is showing, the internal fan should be running and the quartz halogen bulb will go on as needed to heat the sample to the desired temperatures.

If the screen does not change back and forth between the two screens when the RUN/STOP key is pressed, shut the instrument off and unplug the power cord for 30 seconds to 1 minute. Then plug the cord back in and turn the instrument back on. Remember, the protocols set at CYCLE 1, HOLD 1, and LINK 1 will reset to factory preset values if the power is turned off. The remainder of the protocols will remain at their last set values even after power loss.

If the problem persists, please call our service department at the appropriate number listed at the beginning of this section.

Q. When RUN is pressed, the fan runs but the temperature does not increase?

A. If the RUN/STOP key has been pressed and the display shows the "RUN" screen, but the instrument is not heating up, first check the values of the protocol entered to ensure the proper temperature shows on the running protocol. If the proper values are displayed it is possible that the quartz halogen heating bulb has burned out. Check the chamber for light before replacing the bulb. If the bulb has burned out, unplug the machine and replace the bulb following instructions shown in the Service and Maintenance section.

If the problem continues, it is possible the thermal fuse in the instrument needs to be replaced. Unplug the machine and follow the thermal fuse replacement instructions shown in the Service and Maintenance section of this manual. If the problem persists, please call our service department at the appropriate number listed at the beginning of this section.

Q. There is unusual noise coming from the machine?

A. First, check underneath the instrument to verify that the air intake fan is free of obstruction. If there are no visible obstructions, go to a standard cycle protocol, put on protective eye wear, remove the front module and press RUN. When the instrument starts, the heating lamp will illuminate the chamber, allowing you to check for debris or obstructions.

<< Do not reach into the instrument while it is running. >>

If an obstruction is seen, unplug the machine and remove the instrument (see instructions for removal in light bulb replacement in Service and Maintenance). When the cover is up and the chamber is accessible, check the

cover for obstructions or debris. If the interior requires cleaning, use only water or water-based cleaners. **Care must be taken not to bend or harm the thermocouple probe,** which looks like a small wire protruding 1/2" (1.25 cm) into the chamber from the side wall.

If there are no obstructions in the chamber, turn off the instrument, unplug the power cord, lay the instrument on its side, and using pencil or pen, carefully turn the lower fan blade; **be very careful not to bend the lower blade**. Check the lower fan blade for contact with the fan guard. If the lower fan blade has one blade which contacts the guard, carefully use the pencil or pen to gently bend the contacting blade slightly up. If anything more than a very slight contact is occurring, please call our service department at the appropriate number listed at the beginning of this section.

If no problem is found in the lower fan blade area and the noise problem persists, please call our service department at the appropriate number listed at the beginning of this section.

Q. The machine is slow to heat up?

A. If the instrument is taking an excessively long time to reach a set temperature, it is possible there is an air leak from the reaction chamber. First, check to be certain that all three of the sample modules are in place and firmly seated. It is not necessary for all of the modules to contain sample tubes, but the instrument cannot operate correctly without all three modules in place.

If all modules are seated correctly, inspect the perimeter of the instrument top for fit, and check the four corner screws securing the instrument top to ensure they are tightened snugly. If the instrument top is not aligned correctly, undo the top and reset in place following the instruction for top removal and replacement in the Service and Maintenance section of this manual.

The other potential source for a leak is the solenoid operated door located at the top of the rear air duct. If the door does not completely close when the instrument is attempting to reach a denaturation temperature, look around the door for any obstructions. If the door does not close and no obstructions are apparent, please call our service department at the appropriate number listed at the beginning of this section.

Q. The machine is slow to cool down (>15 sec. from denaturation to annealing temperatures)?

A. The RapidCycler requires an unobstructed supply of room temperature air around the entire base of the machine. Setting the machine too close to a wall or surrounding it with books or other objects cuts off the air supply and slows the cooling down. It is also important for the air outlet at the top rear of the machine to be unobstructed.

If after clearing all obstructions the cool down is still slow, run a cycle protocol and watch the solenoid activated door during the transition between denaturation and annealing. The door is visible by looking through the grill at the top rear of the instrument. If the door does not open roughly 2 cm, or if very little hot air is being vented from the duct, please call our service department at the appropriate number listed at the beginning of this section.

Q. The machine cycle normally but reactions are not working?

A. There are numerous factors influencing the outcome of reactions including reagents, reaction kinetics, and secondary DNA or RNA structure. The most common mistakes affecting the outcome of a reaction have been outlined in the Rapid Cyclist Vol. 2, located in the back of this manual.

While Idaho Technology does not warrant the RapidCycler for any specific biochemical reaction, technical assistance for the instrument is available. For assistance, call our service department at the appropriate number listed at the beginning of this section.

Keep in mind that of the many factors influencing the outcome of a reaction, the RapidCycler affects only one - namely the temperature profile. If the temperature on the display seems to indicate the sample is being cycled as expected, you can be confident that the reading accurately reflects the sample temperature.

Service and Maintenance

Phone numbers to call for service problems:
US and Canada: 1-800-735-6544
Outside the US: 1 (801) 736-6354
Fax: 1 (801) 588-0507

E-mail addresses:
Idaho Technology: it@idahotech.com
User's Group: lightcycler@idahotech.com

Web address:
www.idahotech.com

LIGHT BULB REPLACEMENT

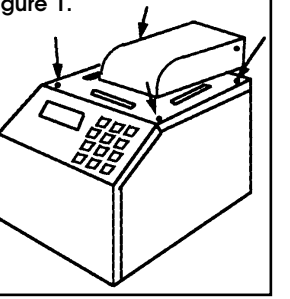
Tools & supplies needed:
Flat blade screwdriver
Replacement Bulb
(Ushio 500 W Mini-Candella halogen bulb)

1. Turn off the power switch on the back of the instrument. Unplug instrument. If the instrument has been recently in operation, wait for approximately five minutes for the light bulb to cool.

<< NEVER ATTEMPT TO REMOVE A HOT BULB >>

2. Completely loosen the four top corner screws. (Figure 1) These screws are "captive" style screws and do not come completely out of the instrument top, but can be completely loosened in place.

Figure 1.



3. Lift the back of the top duct straight up.(Figure 2). The top of the instrument and the top duct should lift up

approximately 7/16" (1 cm). If the instrument top does not lift up, check the four corner screws to ensure they are completely loose. If the top still does not easily lift up, gently pry up the back of the instrument top near the back duct. (Figure 3).

Figure 2.

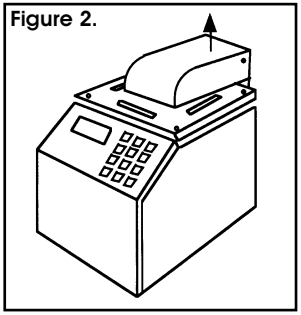
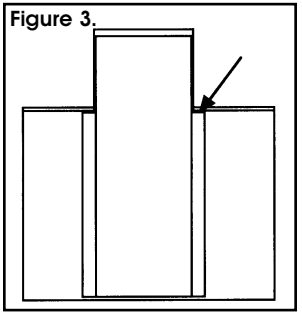


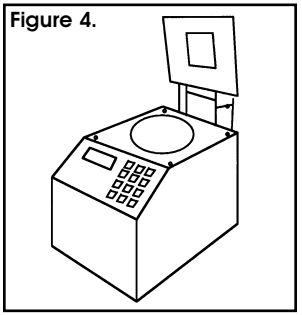
Figure 3.



4. After the instrument top lifts straight up, raise the front of the instrument top up and back to allow access to the bulb. (Figure 4).

5. Carefully check the bulb to ensure it is cool. Unscrew and remove the old bulb, being careful to not break the bulb.

Figure 4.



6. Insert new bulb. Do not touch the glass portion of the new light bulb with bare hands. Use protective liner included with bulb.

7. While the top is open, check the chamber for foreign materials. If you clean the chamber for any reason, only water or water-based cleaners should be used. Care must be taken not to bend or harm the thermocouple probe, which looks like a small wire sticking about 1/2" (1.25 cm) into the chamber from the side wall. It should be sticking straight into the chamber and should not be disturbed.

8. Lift and tip the instrument cover back to a horizontal position. Then, holding the top level, carefully align the top in place and press down. There is an electrical plug inside the top cover which must mate into contacts in the instrument frame. If the top does not fit in place easily, do not force. Lift up on the cover, realign, and press.

9. Once proper fit is established, the four corner screws should be retightened. Do not over-tighten the screws.

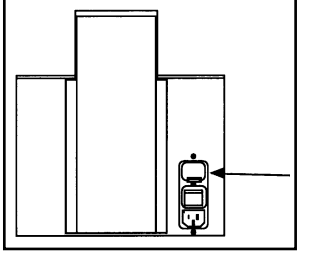
10. Plug instrument back in. Turn switch back on. Check for proper operation of instrument. If problems persist, call our service department.

ELECTRIC FUSE REPLACEMENT

Tools & supplies needed:
Flat blade screwdriver
Replacement fuses

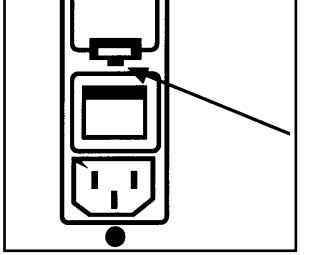
1. Turn off the power switch on the back of the instrument. Unplug instrument. Locate fused switch on back of instrument. (Figure 1)

Figure 1.



2. Insert a small flat bladed screwdriver in fuse tray release slot (Figure 2) and gently lift up. This will allow the fuse tray to be removed.

Figure 2.



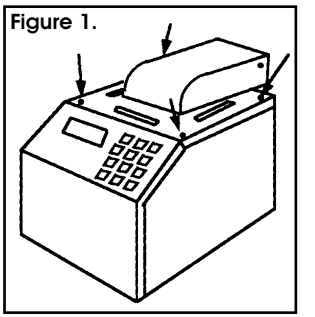
3. Replace fuses with appropriately sized new fuses. Fuse size and style located on the instrument tag on back of instrument. Install fuse tray.

4. Plug instrument back in. Turn switch back on. Check for proper operation of instrument. If problems persist, call our service department at (800) 524-6354.

THERMAL FUSE REPLACEMENT

Tools & supplies needed:
Flat blade screwdriver
5/64" hex wrench
replacement thermal fuse

Figure 1.



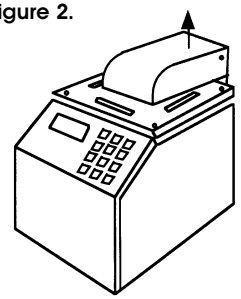
1. Turn off the power switch on the back of the instrument. Unplug instrument. If the instrument has been recently in operation, wait for approximately five minutes for the light bulb to cool.

<< NEVER ATTEMPT TO REMOVE A HOT BULB >>

2. Completely loosen the four top corner screws. (Figure 1) These screws are "captive" style screws and do not come completely out of the instrument top, but can be completely loosened in place.

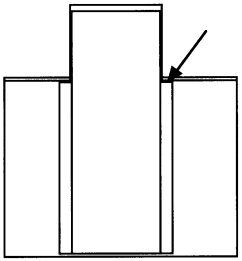
3. Lift the back of the top duct straight up.(Figure 2). The top of the instrument and the top duct should lift up approximately 7/16" (1 cm). If the instrument top does not lift up, check the four corner screws to ensure they are completely loose. If the top still does not easily lift up, gently pry up the back of the instrument top near the back duct. (Figure 3).

Figure 2.



4. After the instrument top lifts straight up, raise the front of the instrument top up and back to allow access to the bulb. (Figure 4).

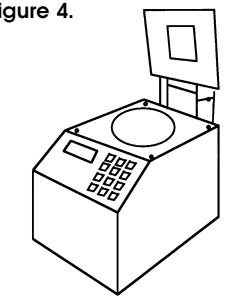
Figure 3.



5. Carefully check the bulb to ensure it is cool. Unscrew and remove the bulb and set it aside, being careful to not break the bulb. Do not touch the glass portion of the light bulb with bare hands.

6. The thermal fuse is inside the top duct (Fig 5). Unplug the two wires connected to the thermal fuse. These are regular slip spade connections and should be relatively easy to remove.

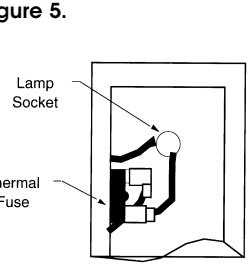
Figure 4.



The thermal fuse is connected to the duct with a hex head screw. A 5/64" hex wrench is needed to remove the screw. Remove the screw and remove the tripped thermal fuse. Insert the new fuse in the same location as the old fuse, and replace and tighten the screw. Reconnect the wires to the thermal fuse, and ensure that all wiring is placed correctly.

7. While the top is open, check the chamber for foreign materials. If you clean the chamber for any reason, only water or water-based cleaners should be used. Care must be taken not to bend or harm the thermocouple probe, which looks like a small wire sticking about 1/2" (1.25 cm) into the chamber from the side wall. It should be sticking straight into the chamber and should not be disturbed.

Figure 5.



8. Replace the bulb. Do not touch the glass portion of the light bulb with bare hands.

9. Lift and tip the instrument cover back to a horizontal position. Then, holding the top level, carefully align the top in place and press down. There is an electrical plug inside the top cover which must mate into contacts in the instrument frame. If the top does not fit in place easily, do not force. Lift up on the cover, realign, and press.

10. Once proper fit is established, the four corner screws should be retightened. Do not over-tighten the screws.

11. Plug instrument back in. Turn switch back on. Check for proper operation of instrument. If problems persist, call our service department at (800) 524-6354.

A PERIODIC MAINTENANCE LIST

DAILY

1. Make sure power switch is off after use (not required but recommended.

2. Make sure nothing is underneath the machine blocking the air intake. Look under the instrument to inspect the fan guard on the bottom of the instrument to ensure there is nothing blocking the air flow.

MONTHLY

1. Inspect chamber for debris.

Remove the four screws that hold the top down. Lift the top straight up about one inch, then swing towards the back of the machine, being careful not to touch the halogen bulb. Make sure there are no broken tubes or debris on the foam.

2. Inspect the thermocouple for damage.

The thermocouple protrudes horizontally from the chamber wall halfway between the top and bottom of the chamber at the 5 o'clock position.

3.Inspect halogen bulb for debris and darkening around the mount.

Slight discoloration around the base of the halogen bulb is normal. Using a dust free cloth, remove any dust or lint that may have collected around the bulb mount. Do not touch the bulb with bare fingers as any residue can shorten the bulb life.

MONTHLY MAINTENANCE, CONTINUED.

1. Inspect fan blade for fatigue at collar attachment point and tighten set screw.

Inspect the area where the fan blade attaches to the mounting collar for any bending or cracking. Check the tightness of the set screw in the fan collar.

2. Inspect and clean chamber including fan blade, foam and modules.

Wipe the entire chamber down with a damp cloth (light soap and water) including the chamber fan blade. Be careful not to bend the thermocouple.

3. Inspect the condition of the duct foam and the door foam

Inspect the foil-covered foam and make sure it is not beginning to peel off the duct sides or top. Make sure the black high temperature foam on the chamber door is not binding with the movement of the door.

4. Clean Keypad with damp cloth.

On the 1002 RapidCycler, wipe the keypad and display area clean with a damp cloth. On the 1605 Air Thermo-cycler use a dry cloth.

EVERY SIX MONTHS

1. Inspect lower (cooling) fan blade and dust if necessary.

Lay the machine on its side and inspect the lower cooling fan and fan guard for anything blocking the air path. If necessary, remove the four screws on the fan guard and wipe the fan blade to remove excessive dust. Also ensure that there is nothing rubbing on the fan blade and it does not hit anything. REPHRASE THIS!

2. Tighten all exterior screws and clean all surfaces.

Using the hex driver supplied with the start-up kit, tighten all of the exterior screws and wipe the surface of the instrument with a damp cloth.

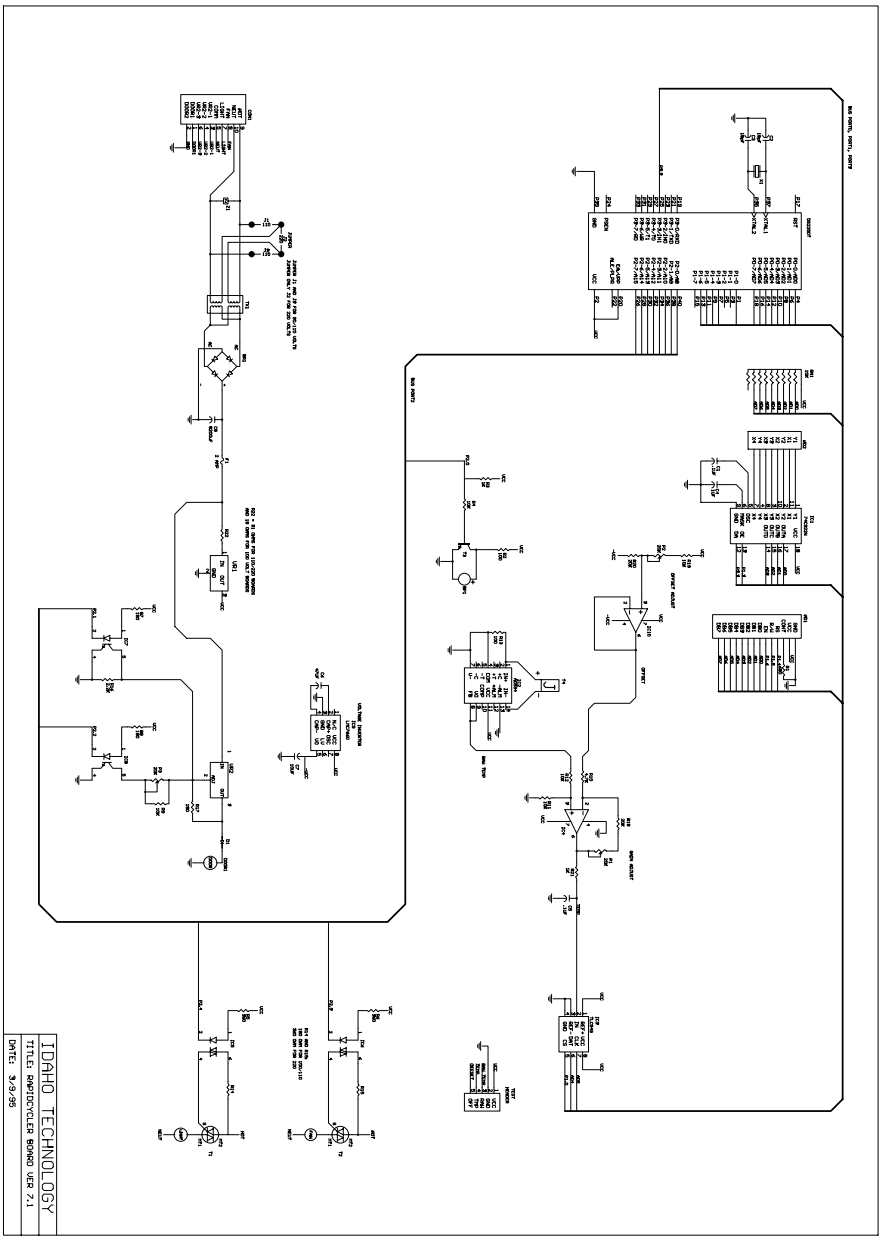
3. Tighten thermal fuse screw.

Remove the four screws that hold the top down. Lift the top straight up about one inch, then swing towards the back of the machine being careful not to touch the halogen bulb. With the hex driver, tighten the screw holding the ther-

mal fuse (Brown rectangle with two wire connectors) located next to the bulb mount on the duct sidewall.

4. Inspect door motor and hinge for friction

Remove the four screws on the rear duct and swing it up. Move the door hinge assembly and make sure that it moves freely and does not bind. If it binds on the leverage arm oil the connecting points with light machine oil - DO NOT OIL THE NYLON HINGE.


IDAHO TECHNOLOGY
TITLE: RAPIDCYCLER BOARD VER 7.1
DATE: 3/9/95

Warranty and Upgrades

A Warranty

Idaho Technology warrants the RapidCycler and related equipment for a period of one year from the date of purchase. If problems occur with your machine, a replacement machine will be shipped immediately via next day air.

In the event of a failure, please call us at 800-735-6544 to arrange for the return, repair and temporary replacement of your machine.

B Upgrades

Because of the modular nature of the Idaho Technology RapidCycler, it will be possible to upgrade the performance of both the hardware and software in the future. Any such upgrades will be offered at low cost and zero downtime.

Articles

Automated polymerase chain reaction in capillary tubes with hot air **Pg. 48**

Nucleic Acids Research, Vol. 17, No. 11, 4353-4357 (1989)
C.T.Wittwer, G.C.Fillmore and D.R.Hillyard
University of Utah Medical School

Minimizing the time required for DNA amplification by efficient heat transfer to small samples **Pg. 54**

Analytical Biochemistry,
Vol. 186, 328-331 (1990)

Carl T. Wittwer, G. Chris Fillmore, and David J. Garling
Department of Pathology University of Utah Medical School,
and Associated Regional and University Pathologists

Rapid cycle DNA amplification: time and temperature optimization **Pg. 63**

BioTechniques, Vol. 10, No.1, 76-83 (1991).
Carl T. Wittwer and David J. Garling
University of Utah Medical School

Automated polymerase chain reaction in capillary tubes with hot air

Nucleic Acids Research, Vol. 17, No. 11, 4353-4357 (1989)
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University of Utah Medical School

ABSTRACT

We describe a simple, compact, inexpensive thermal cycler that can be used for the polymerase chain reaction. Based on heat transfer with air to samples in sealed capillary tubes, the apparatus resembles a recirculating hair dryer. The temperature is regulated via thermocouple input to a programmable set-point process controller that provides proportional output to a solid state relay controlling a heating coil. For efficient cooling after the denaturation step, the controller activates a solenoid that opens a door to vent hot air and allows cool air to enter. Temperature-time profiles and amplification results approximate those obtained using water baths and microfuge tubes.

INTRODUCTION

Cyclic DNA amplification using a thermostable DNA polymerase allows automated amplification of primer specific DNA, widely known as the "polymerase chain reaction" (1,2). Automation requires repetitive temperature cycling. Commercial programmable heat blocks are available and low cost machines using water baths with fluidic switching (3) or mechanical transfer (4) have been described. Instead of heat transfer from metal blocks or water through high thermal resistance plastic microfuge tubes we describe a device that uses hot air for temperature control of samples in thin glass capillary tubes.

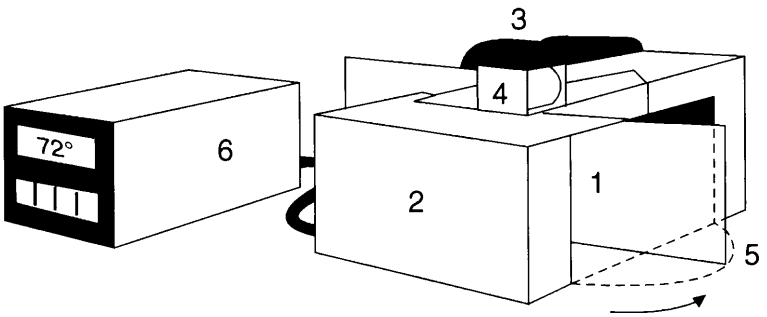


Figure 1. Drawing of the capillary tube, hot air DNA amplifier. I) reaction chamber where a removable stand for capillary tubes can be placed, 2) aluminum housing, 3) air blower, 4) solenoid mechanically coupled to open door on activation, 5) door, normally held closed with a spring, 6) temperature controller.

MATERIALS AND METHODS

The device for temperature cycling is a closed-loop hot air chamber resembling a recirculating hair dryer (Fig. 1 and 2). The heating element is a 1000 W (125 VAC) nichrome wire coil (Johnstone Supply, Portland Oregon) wound around a mica support. The heating coil is activated via a 25 A, 125 VAC solid state relay (Crydom D1225, available as Omega SSR 240 D25 through Omega Engineering Inc, Stamford, CT), connected to a Partlow MIC-6000 proportional temperature controller (available through Omega as the CN8600 process controller) with thermocouple input and at least one SSR driver and one relay output. The relay output controls a solenoid (Dormeyer 2A173, Chicago, IL) mechanically coupled to open a door on activation that interrupts the recirculating hot air and introduces ambient-temperature air during the cool-down portion of each cycle. The door piv-

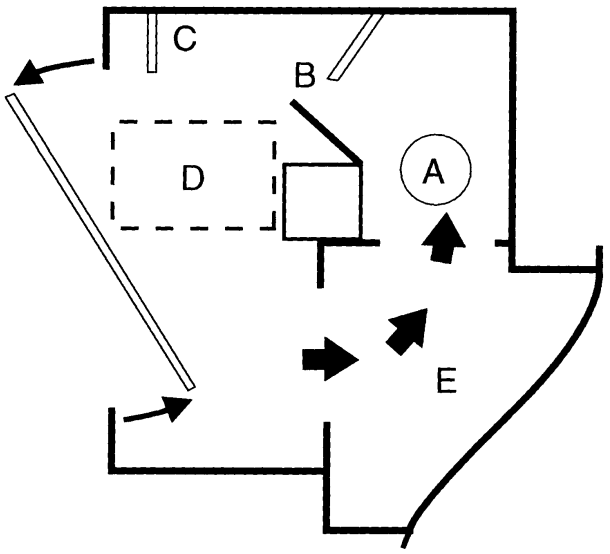
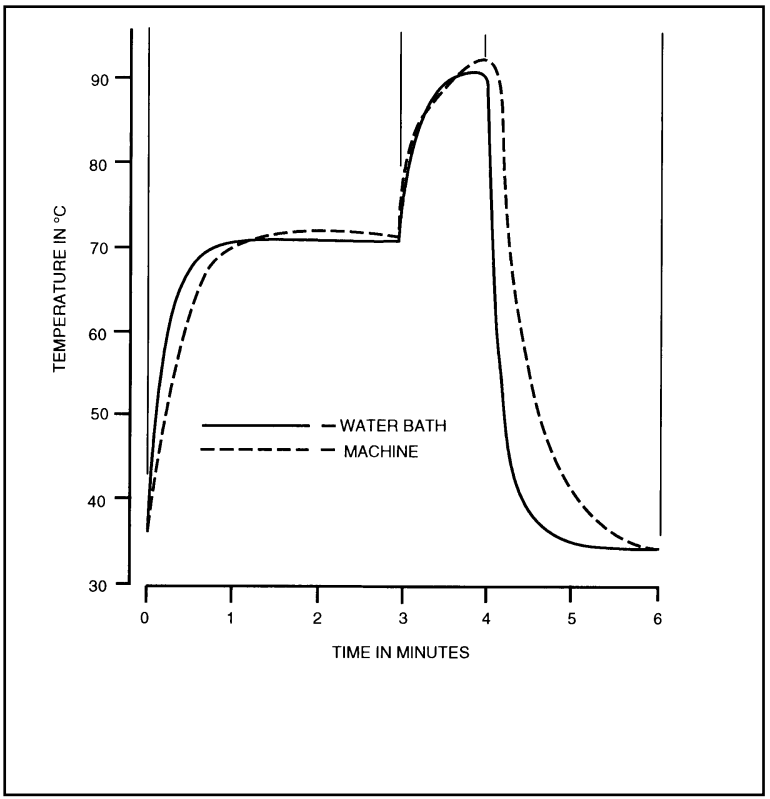


Figure 2. Scale diagram of the amplifier. A) heating coil connected to the controller via a solid state relay, B) baffles to uniformly mix the hot air, C) thermocouple leads connected to controller, D) reaction chamber, E) air blower.

ots on a central axis and is normally held shut with a spring attached to a cam along the central axis. Baffles are placed downstream of the heating coil to mix the air efficiently before it reaches the sample compartment. Air is circulated through the system with an "in-line" 75 cubic feet per minute air blower (Fasco B75, Cassville, MO). Temperature monitoring during routine operation of the cycler is achieved by a 30-gauge iron-constantan "J-type" thermocouple placed just before the sample compartment in the air stream and connected to the temperature controller. The sample compartment is a 5 cm wide x 5 cm long x 10 cm high chamber accessible by manually opening the solenoid-controlled door. The housing of the apparatus is formed from aluminum sheeting.

The polymerase chain reaction was run in a 100 μl volume with 1 ug template DNA, 1.5 mM of each deoxynucleotide, 50 pmol of each oligonucleotide primer and 10% dimethyl sulfoxide in a reaction buffer consisting of 17 mM ammonium sulfate, 67 mM Tris-HCl (pH 8.8 at 25° C), 6.7 mM magnesium chloride, 10 mM beta-mercaptoethanol, 6.7 uM EDTA, and 170 ug/ml bovine serum albumin (5). After denaturing the reaction mixture at 94° C for 5 minutes, 1 unit of Thermus aquaticus polymerase (Taq polymerase - Stratagene, La Jolla, CA) was added, the samples placed in 10 cm long, thin-walled capillary tubes (Kimble, Kimax 34500), and the ends fused with an oxygen-propane torch so that an air bubble was present on both sides of the sample. The capillary tubes were placed vertically in a holder constructed of 1 mm thick "prepunched perfboard" (Radio Shack, Fort Worth TX). The mixture was cycled 30 times through denaturation (94° C - 1 min), annealing (37° C - 2 min) and elongation (70° C - 3 min) steps. Temperature moni-



toring within the capillary tubes was done with 30-gauge J-type thermocouple wire placed in 100 μl of deionized water and connected to a thermocouple meter (Precision Digital PD710, Watertown, MA). Amplification products (5/100 μl) were fractionated by electrophoresis on a 1.5% agarose gel.

RESULTS AND DISCUSSION

The temperature profile of a sample in the cycling apparatus was compared to that obtained by manually transferring microfuge tubes between water baths. Response times at each phase of the thermal cycle were roughly equivalent (Fig. 3). The temperature response of samples transferred between water baths is limited only by the heat conduction properties of the microfuge tube. The response times of commercial machines is also limited by the heat capacity of their metal heating/cooling blocks. The air cycler has the advantage that heat transfer occurs through a low heat capacity medium (air) that can be warmed very rapidly. The response time for sample cooling depends strongly on the heat capacity of the system materials. The current cycler was constructed from materials to approximate the heating response of microfuge tubes transferred between water baths. Although the current performance profile seems perfectly adequate, thinner housing material and an external fan motor (with only the blades and shaft exposed to the circulating hot air) could give even faster response

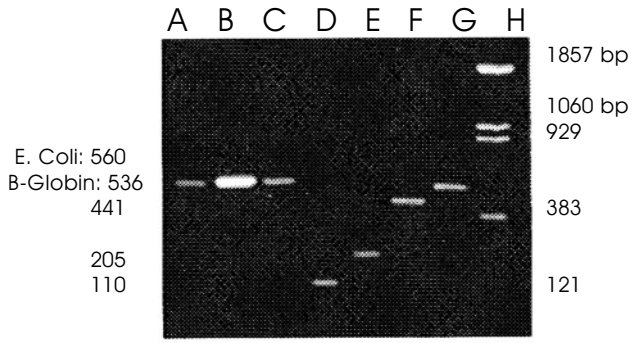


Figure 4. Ethidium bromide stained amplification products. Lane A shows the product of amplification in microfuge tubes manually transferred between water baths for comparison to the hot air amplifier in lanes B-G. Lanes A-C) 560 bp fragment of E. coli DNA defined by primers TGAATCTG-TACTCTGATGTAAC and CACTAATAGCAAGAGGGTACTCAG covering a portion of the regulatory region for pyelonephritis-associated pili (6). An asymmetric amplification (50 pmol of one primer and 0.5 pmol of the other is shown in lane C. Lanes D-G) amplification products of 4 different combinations of the human ß-globin gene primers PC03, PC04 (7), KM29, and RS42 (8). Lane H) BstN I digest of pBR322 DNA size markers (0.5 ug).

times. This might allow optimization of denaturation, annealing, and elongation steps in terms of time and temperature, and shorten the "ramp" times between temperatures. This could decrease the time required for a complete amplification, as well as allow specific study of annealing, denaturation and enzyme kinetics within a polymerase chain reaction protocol.

Because of the low heat capacity of air, thin glass capillary tubes were used to contain the samples rather than plastic tubes. Attempts to amplify DNA in various plastic tubes with the air cycler were unsuccessful and temperature profiles were sluggish. Capillary tubes require a torch to seal the ends, but this can be readily achieved with only minimal practice. In order to obtain adequate temperature homogeneity within the sample compartment, baffles were installed between the heating coil and the samples. With the cycler set at a constant temperature (from 70 to 95° C), simple structural baffles decreased the temperature variation observed throughout the sample compartment from about 10° C, to 2° C. This can be improved further by more complicated baffles if necessary .

Amplification products obtained with the device are qualitatively and quantitatively similar to those observed after manual water bath cycling (Fig. 4). We have used the apparatus to amplify both bacterial and human genomic DNA. Best results have been obtained with denaturation temperatures between 90 and 94° C. At temperatures above 94° C, amplifications are often not successful, apparently due to enzyme denaturation. This may result from faster equilibration of the sample at high temperature with the air cycler compared to other machines. This would effectively expose the polymerase to the high denaturation temperature for a longer period of time.

ACKNOWLEDGEMENTS

We thank Mr. Charles Schaemal for design and construction assistance and Dr. David Low for the E. coli probes and DNA.

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Minimizing the time required for DNA amplification by efficient heat transfer to small samples

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Hot-air temperature cycling of 1- to l0 μl samples in glass capillary tubes can amplify DNA by the polymerase chain reaction in 15 min or less. A rapid temperature cycler of low thermal mass was constructed to change sample temperatures among denaturation, annealing, and elongation segments in a few seconds. After 30 cycles of 30 s each, a 536-bp, B-globin fragment of human genomic DNA was easily visualized with ethidium bromide on agarose gels. With rapid cycling, amplification yield depended on polymerase concentration. The time required for DNA amplification can be markedly reduced from prevailing protocols if appropriate equipment and sample containers are used for rapid heat transfer to the sample. 1990 Academic Press, Inc.

The minimum time required for DNA amplification by the polymerase chain reaction (1,2) has not been rigorously investigated. No systematic study of optimal times for annealing, elongation, and denaturation is available because no device has been able to change the sample temperature quickly enough to make such study meaningful. Commercial instruments spend a significant amount of time changing the sample temperature (3) .

A number of commercial cyclers use aluminum blocks and microfuge tubes to cycle temperature for the polymerase chain reaction. Standard protocols for a 30-cycle amplification are usually 2-6 h in length, and a large fraction of this time is spent heating and cooling the sample. Time is required both to bring the

Abbreviations used: DMSo, dimethyl sulfoxide;
dNTP, deoxynucleoside triphosphate.

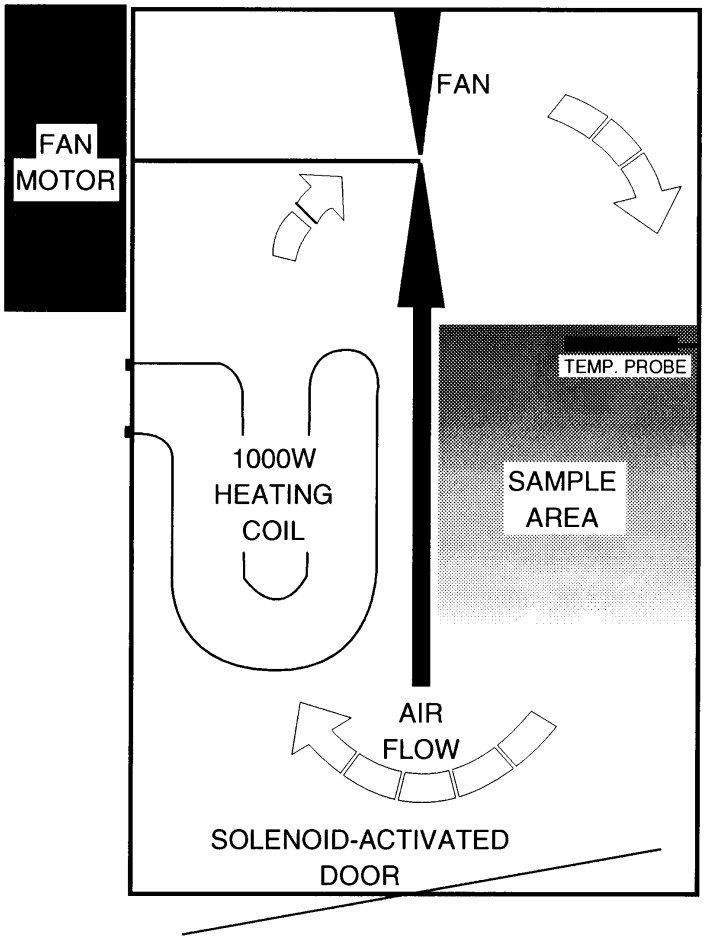


Figure 1. Diagram of the rapid DNA amplifier. A horizontal section through the air cycler is shown. Recirculating air is heated by a 1000W coil and mixed by fan blades while a thermocouple monitors the airstream temperature in the sample area and provides input to the proportional controller. 'The fan motor is mounted outside of the airstream to decrease the thermal mass of the system. A solenoid-activated door opens for rapid cooling between denaturation and annealing stages. The air chamber is 10 cm in height, 10 cm in width, and 20 cm in depth. Samples are contained in glass capillary tubes that are placed vertically in the sample area of the cycler.

sample block to temperature and to transfer heat to the sample through a microfuge tube (3). These systems have limited response times because of high heat capacity of the metal blocks and low heat transfer through thick plastic microfuge tubes.

An alternative approach for thermal cycling uses air for heat transfer (4-6) and contains samples in thin glass capillary tubes (5,6). With forced-air heating and 100 - μl samples, temperature profiles similar to those obtained by transferring microfuge tubes between water baths can be obtained (6). None of the air-cycling systems have capitalized on the potential for even faster response times. Our objective was to see if amplification times could be significantly reduced by decreasing both the heat capacity of the air-cycling system and the sample volume.

MATERIALS AND METHODS

The rapid air cycler is based on a previously described design (6). Its thermal mass was reduced by using thin aluminum sheeting for the housing and placing the fan motor (3000 rpm, 1/40 HP, ball bearing C-frame motor No. 4M080, Grainger, Salt Lake City, UT) outside of the airstream (Fig. 1). The fan blades (3.5-in aluminum No. 2C951, Grainger) were placed downstream from the heating coil to mix the heated air before reaching the samples. Up to 30 capillary tubes could easily be placed in the sample compartment. The sensing thermocouple, proportional temperature controller, and solenoid-activated door (for cooling with ambient air) have been previously described (6).

The proportional controller was programmed to obtain desired cycle times of 20, 30, 60,120, and 180 s. The temperature response of the sample was recorded from the analog output of a BAT-12 temperature monitor (Sensortek, Clifton, NJ) connected to a miniature thermocouple (IT-23, 0.005-s time constant, Sensortek) placed within a 10-μl sample in a microcapillary tube (KIMAX 46485-1, Kimble, Vineland, NJ).

DNA amplification was performed with 50 mM Tris, pH 8.5 (at 25°C), 3 mM MgC12, 20 mM KCI, 500 ug/ml bovine serum albumin, 5% DMSO, 0.5 ,uM each of the human B-globin genomic primers KM29 and RS42 (7), 0.5 mM of each dNTP, 50 ng of placental human genomic DNA, and 0.1 -0.8 U of Taq polymerase/10 μl. One unit (U) of polymerase activity was the amount of enzyme required to incorporate 10 nmol of [3H]dTTP in 30 min at 80°C as defined by the manufacturer (Stratagene, La Jolla, CA). All other reagents were from Sigma (St. Louis, MO). The DMSO, KCI, albumin, and MgC12 concentrations were optimized by individual titrations for amplifying the KM29/RS42 primer pair region of genomic DNA. Samples (10 μl) were placed in 8-cm capillary tubes (KIMAX 46485-1) and the ends fused with an oxygen-propane torch. Samples of 100 μl were placed in larger diameter l0-cm tubes (KIMAX 34500). The capillary tubes were placed vertically in the sample area of the rapid air cycler. The temperature of the samples was cycled 30 or 40 times through denaturation, annealing, and elongation steps of 90-92°C, 50-55°C, and 71-73°C, respectively, for the times indicated in individual experiments. Amplification products (9 μl unless indicated otherwise) were fractionated by electrophoresis on a 1.5% agarose gel and visualized with ethidium bromide and uv transillumination.

RESULTS

The temperature response of 10-u1 samples during 30- and 60-s cycles of the rapid air cycler is shown in Fig. 2 and Fig. 3, respectively. The annealing segment of each temperature profile is a spike corresponding to cooling of the sample with ambient air. The denaturation segment of the 30-s cycle is also a spike with very little time spent at the high temperature. The major difference between the 30- and the 60-s cycles is the length of the elongation segment. Some oscillation

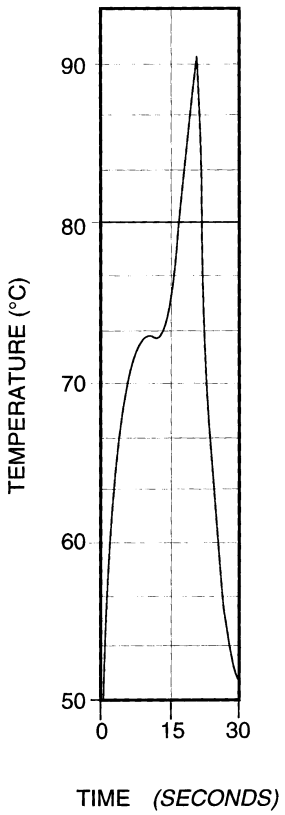


Figure 2. Sample temperature during a 30-s cycle. Chart recording of the analog output of a BAT-12 temperature monitor with an IT-23 thermocouple probe (time constant 0.005 s, Sensortek, Clifton NJ). The thermocouple was placed in 10 μl of water within a microcapillary tube.

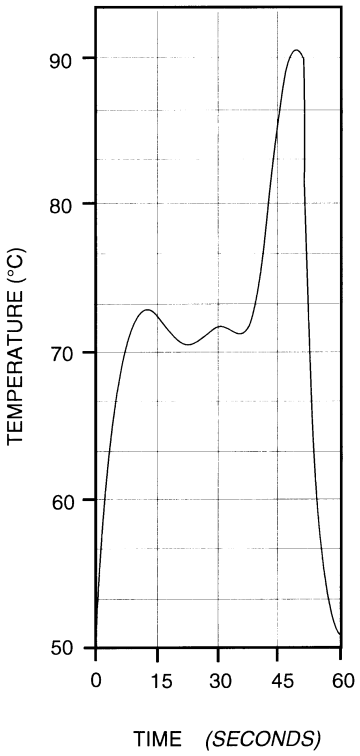


Figure 3. Sample temperature during a 60-s cycle. Conditions of measurement were as described in Fig. 2.

Figure 4. Micro DNA amplification. Ethidium bromide-stained amplification products of human genomic DNA delimited by the ß-globin primers KM29 and RS42(7). From 1- to l00-μl samples were amplified in capillary tubes using 40 cycles of amplification (1 min at 90°C, 1 min at 55°C, 1 min at 72°C) and the air cycler previously described (6). The resulting product, 1 to 10 μl, was applied to a 1.5% agarose gel.

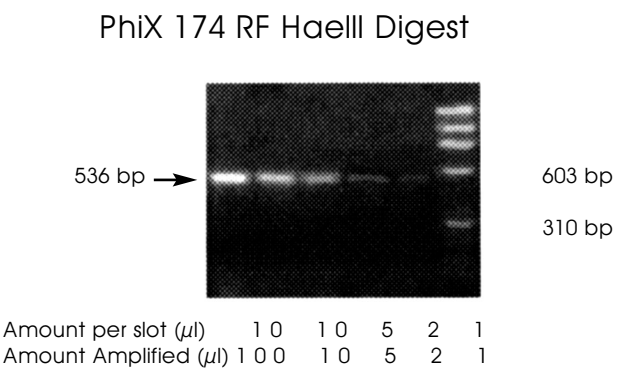


Figure 5. Rapid DNA amplification is dependent on polymerase concentration. The total amplification time was 20 min and consisted of 40 30-s cycles as shown in Fig. 2. The amount of Taq polymerase varied from 0.1 to 0.8 U in each 10-μl sample.

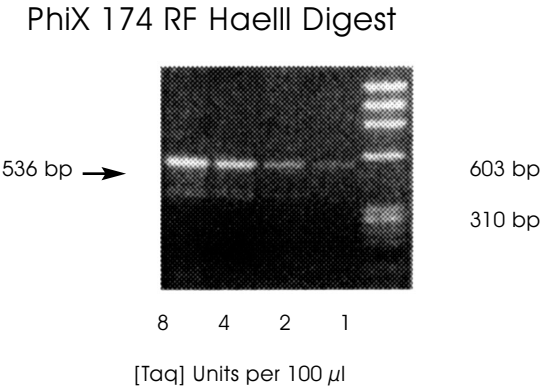
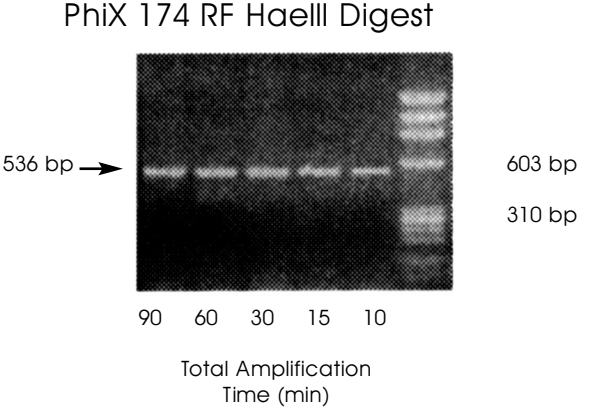


Figure 6. Rapid DNA amplification. Each 10-u1 sample contained 0.8U of Taq polymerase and 30 cycles of amplification were performed.Sample temperature profiles for the 15- and 30-min amplifications are given in Figs. 2 and 3, respectively. Other temperature profiles are described in the text.



around the elongation temperature is evident in the 60-s cycle from the proportional controller. Temperature profiles for the 20s cycle showed only a slight inflection at a sample elongation temperature of 72°C (not shown). The 120-s and 180-s cycles had elongation times twice their denaturation times (not shown).

Samples of 10 μl can be amplified with a yield equivalent to l00 μl samples in the air cycler (Fig. 4). In capillary tubes, the amplification volume can be reduced to 1 μl with the product still detected by ethidium bromide staining in agarose gels.

Gels from rapid amplifications are shown in Figs. 5 and 6. In Fig. 5, the dependence of amplification on polymerase concentration is shown for 30-s cycles. Band intensity is strongly dependent on the amount of polymerase added. Figure 6 shows that although amplification efficiency is reduced with extremely rapid cycling, significant amplification still occurs after a total amplification time of only 10 min. Control samples without template DNA or polymerase did not show visible bands (not shown) .

DISCUSSION

The advantages of an air-cycling system for DNA amplification include simplicity, low cost, and rapid temperature cycling. Air is an ideal heat transfer medium which can change temperature quickly because of its low density. Air can he rapidly mixed with baffles (6) or by a fan (Fig. I) to provide homogeneous temperature exposure over the sample containers. The low thermal conductivity of air requires that air be rapidly blown past the heating coils and sample containers for efficient heat transfer.

Any temperature cycling protocol for DNA amplification can be divided into six segments: three endpoint temperatures and three temperature transitions. Time spent in transition is usually wasted, although theoretically a slow transition between annealing and elongation may be useful for a poorly annealing primer. Transition times after elongation and denaturation have no function; the faster the sample can be cooled after denaturation the better. Rapid cooling after denaturation favors the kinetic process (primer annealing to template/product) over the equilibrium process (product dimerization).

Annealing and denaturation are claimed to occur almost instantaneously once the sample has reached the appropriate temperature (3). Classical kinetic studies on DNA renaturation (8,9) also predict rapid annealing because of the high primer concentration used in DNA amplification. However, to our knowledge, this has not previously been tested. Our results suggest that denaturation

and annealing do in fact occur very quickly in DNA amplification, with good amplification occurring even when the denaturation and annealing segments are reduced to spikes (Figs. 2, 5, and 6). The polymerase chain reaction need not take hours to perform; with appropriate temperature cycling equipment, DNA amplification can occur in minutes.

The ultimate limit of how fast DNA amplification can occur is not answered by this study. The times required for denaturation and annealing are apparently minimal. Primer extension is not instantaneous and the elongation time required depends on the length of the amplified product. Taq polymerase is highly processive with an extension rate of >60 nucleotides/s at 70°C (10). The large effect of polymerase concentration on band intensity with rapid cycling (Fig. 5) suggests that polymerization time becomes the limiting factor at very short cycle times (Fig. 6).

For rapid temperature cycling, the sample container is just as important as the thermal cycler. An optimal sample container should be water-vapor tight and have (i) low thermal mass, (ii) good thermal conductivity, (iii) minimal internal condensation, (iv) easy sample recovery without cross contamination, and (v) no inhibition of DNA amplification. Whatever the container, temperature equilibration will always be achieved faster if the sample volume is small, if the container wall is thin, and if the surface-to-volume ratio of the sample exposed to the container wall is high. Problems with condensation can be reduced by minimizing the free air space surrounding the sample.

Microfuge tubes are kept water-vapor tight by mechanical closure and, if necessary, overlaid mineral oil. Thermal conductivity is poor because of the material and its thickness (ca. 1 mm). Internal condensation can occur if mineral oil is not used and particularly if different parts of the tube are at different temperatures (which depends on the temperature cycler configuration).

In contrast, glass capillary tubes are made vapor tight by flame closure of the ends. They conduct heat to the sample better than microfuge tubes because of decreased wall thickness (ca. 0.2 mm) and a better surface-to-volume ratio. Dead air space can be minimized to prevent significant condensation. Different diameter capillary tubes can be chosen for the sample volume desired.

Decreasing the heat capacity of the cycling system can markedly decrease the total time required for the polymerase chain reaction. In addition, air cycling and miniaturization significantly decrease the cost of DNA amplification. There may be other advantages of rapid cycling; decreased annealing and denaturation times should theoretically reduce nonspecific amplification and polymerase inactivation, respectively.

When the temperature response of the cycler and thermal equilibration of the sample are not limiting, questions about optimal temperatures and times for DNA amplification can be answered to much greater accuracy than before. The physical processes of denaturation and annealing and the enzymatic process of elongation can be specifically studied, without the confounding effects of long transitions between temperatures. This should lead to a more detailed understanding of DNA amplification and improved reaction efficiency and specificity.

ACKNOWLEDGMENT

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Rapid cycle DNA amplification: time and temperature optimization

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ABSTRACT

Rapid temperature cycling with hot air allows rigorous optimization of the times and temperatures required for each stage of the polymerase chain reaction. A thermal cycler based on recirculating hot air was used for rapid temperature control of 10 μl samples in thin glass capillary tubes with the sample temperature monitored by a miniature thermocouple probe. The temperatures and times of denaturation, annealing, and elongation were individually optimized for the amplification of a 536-base pair ß-globin fragment from human genomic DNA. Optimal denaturation at 92°-94°C occurred in less than one second; yield decreased with denaturation times greater than 30 seconds. Annealing for one second or less at 54°-56°C gave the best product specificity and yield. Non-specific amplification was minimized with rapid denaturation to annealing temperature transition (9 seconds) as compared to a longer transition (25 seconds). An elongation temperature of 75°-79° C gave the greatest yield and increased yields were obtained with longer elongation times. Product specificity was improved with rapid air cycling when compared to slower conventional heat block cycling. Rapid thermal control of the temperature-dependent reaction in DNA amplification can improve product specificity significantly while decreasing the required amplification time by an order of magnitude.

INTRODUCTION

Automated in vitro DNA amplification with a heat stable DNA polymerase requires temperature cycling of the sample (11,14). Temperature transition rates in commercial instruments are usually less than 1° C/s when metal blocks or water are used for thermal equilibration and samples are contained in plastic microcentrifuge tubes (10,12). A significant fraction of the cycle time is spent heating and cooling the sample, as opposed to being spent at optimal denaturation, annealing, and elongation temperatures. Extended amplification times of 2-6 hours are common, and long transition times make it difficult to determine opti-

mal temperatures and times for each stage. Instantaneous temperature changes are not possible because of sample, container and cycler heat capacities.

We have recently constructed a rapid cycling system of low heat capacity based on heat transfer by hot air to samples contained in thin glass capillary tubes (20,21). Amplified product from genomic DNA can be easily visualized with ethidium bromide on agarose gels after a total amplification time of 15 min or less (21). The rapid temperature response of this instrument allows systematic study of the times and temperatures required for annealing, elongation, and denaturation in DNA amplification because transition times can be reduced to a minimum.

MATERIALS AND METHODS

DNA amplification was performed in 50 mM Tris, pH 8.5 (25°C), 3 mM MgC12, 20 mM KCl, 500 μg/ml bovine serum albumin, 0.5 μM each of the human ß-globin genomic primers RS42 and KM29 (13), 0.5 mM of each deoxynucleoside triphosphate (dNTP), 2.5% (wt/vol) Ficoll® 400, 50 ng of human genomic DNA, and 0.4 U of Taq polymerase per 10 μl unless specified otherwise. Although 5% dimethyl sulfoxide (DMSO) was used with this primer pair in our previous study (21), it was omitted here because of its reported effect on polymerase activity (4). Tartrazine (l mM) or xylene cyanole (0.02% wt/vol) was sometimes added to the reaction mixture for easy visualization. Ficoll 400 and the indicator dyes could be added to the reaction mixture at the concentrations listed without significantly affecting product yield or specificity. Ten times stock solutions of the primers, dNTPs, and DNA contained 10 mM Tris, pH 8.0 and 0.1 mM EDTA. Human genomic DNA (50 μg/ml) was denatured for 1 min by boiling and then rapidly cooled on ice before use in amplification. One unit of polymerase activity was the amount of enzyme required to incorporate 10 nmol of dNTPs in 30 min at 74°C as defined by the manufacturer (Promega, Madison, WI). All other reagents were from Sigma Chemical (St. Louis, MO).

A single amplification mixture was used for all samples viewed on one gel. Taq polymerase was accurately measured with a 1-μl microcapillary pipet (Microcaps®, Drummond Scientific, Broomall, PA) and diluted in 10 mM Tris, pH 8.5, 100 μg/ml bovine serum albumin if necessary. Samples (10 μl) were placed in 8-cm lengths of microcapillary tubing (KIMAX 46485-1, Kimble, Vineland, NJ), and the ends were sealed with a Bunsen burner. A 1-2-cm column of air on each side of the sample allowed easy sealing (and opening) of the tubes. Thirty cycles of DNA amplification performed in a custom-made hot-air thermal cycler. Inclusion of Ficoll 400 and a dye (tartrazine or xylene cyanole) into the amplification mixture allowed samples to be directly emptied into wells of a l.5% agarose gel (using

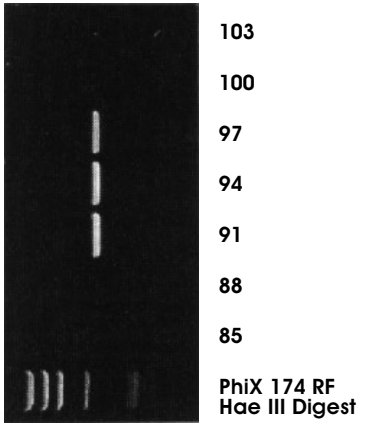

Figure 1. Effect of denaturation temperature on DNA amplification yield. DNA amplification was performed for 30 cycles as described in Materials and Methods except that the denaturation temperature was varied from 85-103 C. Temperatures greater than boiling could be attained because sealed capillary tubes were used for sample containment. The denaturation time at each temperature (+/- 1° C) was 1 sec or less, the total amplification time was 14-16 mln, and the temperature-time profiles approximated that shown in Figure 7D. Ethidium bromide-stained amplification products of human genomic DNA delimited by the beta-globin primers RS42 and KM29 (7) were electrophoresed on a 1.5% agarose gel.

Captrol III, Drummond Scientific), electrophoresed and viewed with ethidium bromide/UV transillumination. All experiments included a control without genomic DNA where no amplification was observed.

The hot-air thermal cycler (20) and modifications necessary for rapid cycling (21) have been previously described. The temperature response of the sample was recorded with a miniature thermocouple (IT-23, 0.005 s time constant, Sensortek, Clifton, NJ). Unless otherwise specified, the times and temperatures of the sample for each amplification stage were as follows: denaturation, 1 s at 92°-94° C; annealing, 1 s at 54°-56°C; and elongation, 10 s at 75°-79 ° C. Transition times were usually as follows: denaturation to annealing (92°-56°C), 9 s; annealing to elongation (56°-75° C), 4 s; and elongation to denaturation (79°-92°C), 5 s.

The hot air cycler uses a solenoid-activated door to allow room temperature air to cool the samples between denaturation and annealing (21). Under normal operation, a low-temperature "spike" occurs at the annealing temperature with a quick rebound to the elongation temperature. With the door open, the airstream and sample temperatures are not in equilibrium with the system (aluminum housing, fan blades, heating coil) so when the door closes, the sample temperature is quickly increased by heat transfer from the system back to the airstream and sample. This has previously prevented study of extended annealing times or low elongation temperatures. This limitation was overcome by only partly opening the solenoid-activated door, resulting in partial air recirculation and a slower denaturation to annealing transition. The transition time was increased from 9 to 20-25 s and the system and sample were in temperature equilibrium when the door closed. This allowed annealing times at 54°-56°C longer than 1 s (Figure 4) and elongation temperatures below 70°C (Figure 5) .

The time or temperature of individual amplification stages was varied systematically as indicated in each figure. The optimum temperature was first determined, and then the effects of varying the time at that temperature were investigated. Finally, rapid cycling was compared to conventional heat block cycling using an identical amplification mixture.

RESULTS

Using the RS42/KM29 primer pair for amplifying a 536-base pair fragment of ß-globin from human genomic DNA, the effects of varying temperatures and times for denaturation, annealing, and elongation were studied. Figure 1 shows that momentary denaturation (<1 s) at 91°-97° C was adequate for DNA amplification. Little amplification occurred with a denaturation temperature below 91°C, presumably because of inadequate strand separation. Above 97° C product amplification was also minimal. Figure 2 shows equivalent amplification with denaturation times from 1-16 s when the denaturation temperature was 92°-94° C. Decreased yield with long denaturation time (>30 s) or high denaturation temperatures may be secondary to polymerase inactivation, or to compromise of other reaction components (dNTP breakdown, albumin coagulation, etc.).

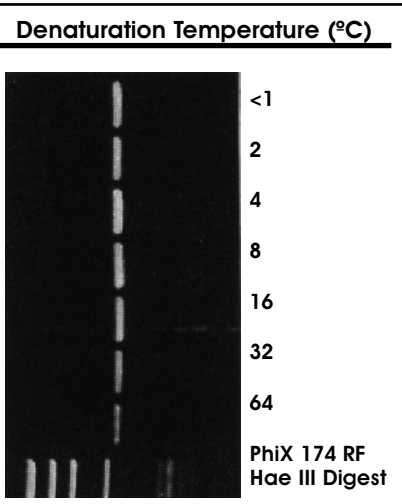

Figure 2. Effect of denaturation time on DNA amplification yield. The denaturation time at 92-94°C was varied from 1-64 sec. Other conditions were as given in Figure 1.

The optimal annealing temperature was about 55° C (Figure 3). Lower annealing temperatures resulted in decreased yield of the desired product and an increase in nonspecific amplification. Little specific amplification occurred at an annealing temperature of 40°C. Although nonspecific amplification was minimized at temperatures > 55° C , desired product yield also decreased, presumably because of incomplete annealing. Figure 4 shows that the shortest possible annealing time (<l s) and the fastest denaturation to annealing transition (9 s) gave the highest yield and the least nonspecific amplification.

The optimal elongation temperature was between 75°-79°C with little amplification above 80°C or below 70°C (Figure 5). Longer elongation times increased product yield as shown in Figure 6, although the increase in yield appeared to plateau after 40-80 s.

Figure 7 compares the 30-cycle amplification product obtained with four different temperature profiles. Conventional heat block/microcentrifuge tube cycling was used in Figure 7A and 7B. The transitions between temperatures were relatively slow and many nonspecific amplification bands were present. Nonspecific amplification was reduced by limiting the time at each temperature (Figure 7B compared to Figure 7A). Using rapid hot air cycling (Figures 7C and 7D) nonspecific amplification was dramatically reduced. Easily visible specific product was apparent using only a 10 s elongation (Figure 7D), although extending the elongation time to 60 s did increase the yield (Figure 7C) .

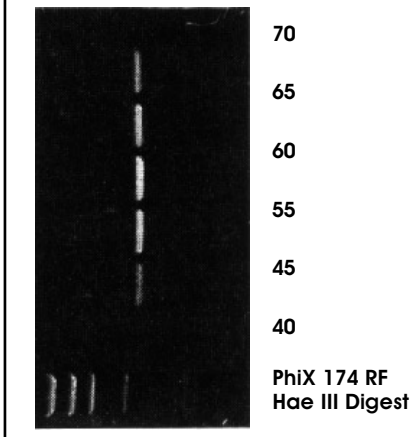

Figure 3. Effect of annealing temperature on DNA amplification yield. Amplification was performed for 30 cycles as described in Materials and Methods except that the annealing temperature was varied from 40 to 70°C. The annealing time at each temperature (+/- 1°C) was <1 sec.

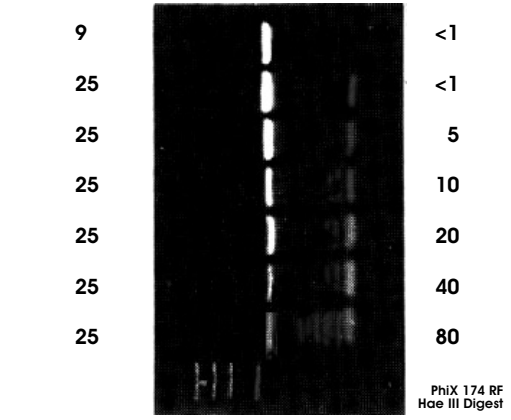

Figure 4. Effect of annealing time, and denaturation to annealing transition time on DNA amplification yield. The denaturation to annealing transition time was either 9 sec (door completely open) or 25 sec (door partially open). The annealing time at 54-56 C° was varied from 1-80 sec. Other conditions were as given in Figure 3.

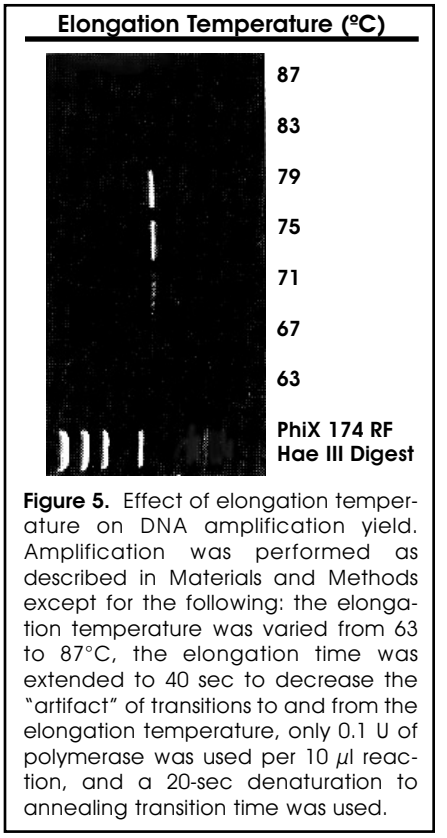

Figure 5. Effect of elongation temperature on DNA amplification yield. Amplification was performed as described in Materials and Methods except for the following: the elongation temperature was varied from 63 to 87°C, the elongation time was extended to 40 sec to decrease the "artifact" of transitions to and from the elongation temperature, only 0.1 U of polymerase was used per 10 μl reaction, and a 20-sec denaturation to annealing transition time was used.

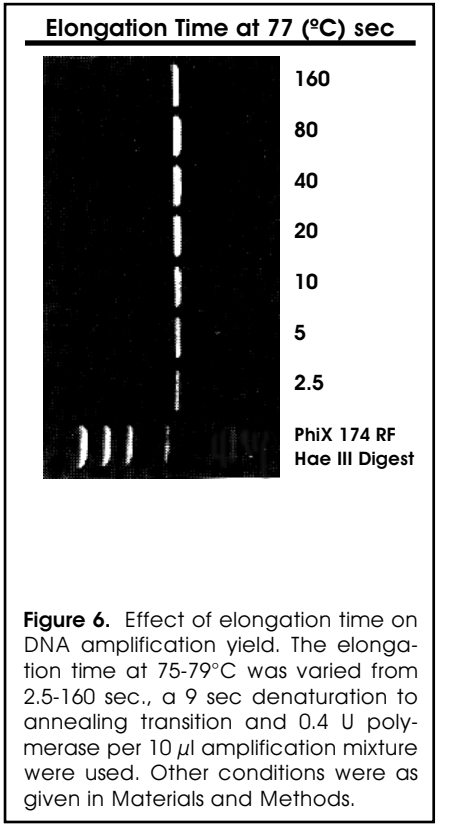

Figure 6. Effect of elongation time on DNA amplification yield. The elongation time at 75-79°C was varied from 2.5-160 sec., a 9 sec denaturation to annealing transition and 0.4 U polymerase per 10 μl amplification mixture were used. Other conditions were as given in Materials and Methods.

DISCUSSION

It is well recognized that DNA amplification is critically dependent on the sample temperature-time profile. However, a precise description of this profile is seldom achieved. Often, heat block temperatures are used instead of actual sample temperatures. The temperature of a 100 μl sample within a microcentrifuge tube in a heat block instrument reportedly lags 20 s behind the heat block temperature (12). In a representative commercial instrument, we found that 35 s were required for the sample to reach the block temperature (Figure 7B and legend). This lag time may vary with the exact position of the tube in the heat block, as uneven heating and cooling have been reported (8,22).

The actual sample temperature can be monitored by a thermocouple probe in the sample. The probe needs to be small enough so that it does not significantly affect the temperature response. The thermocouple should be positioned to accurately reflect the sample temperature, which is presumably homoge-

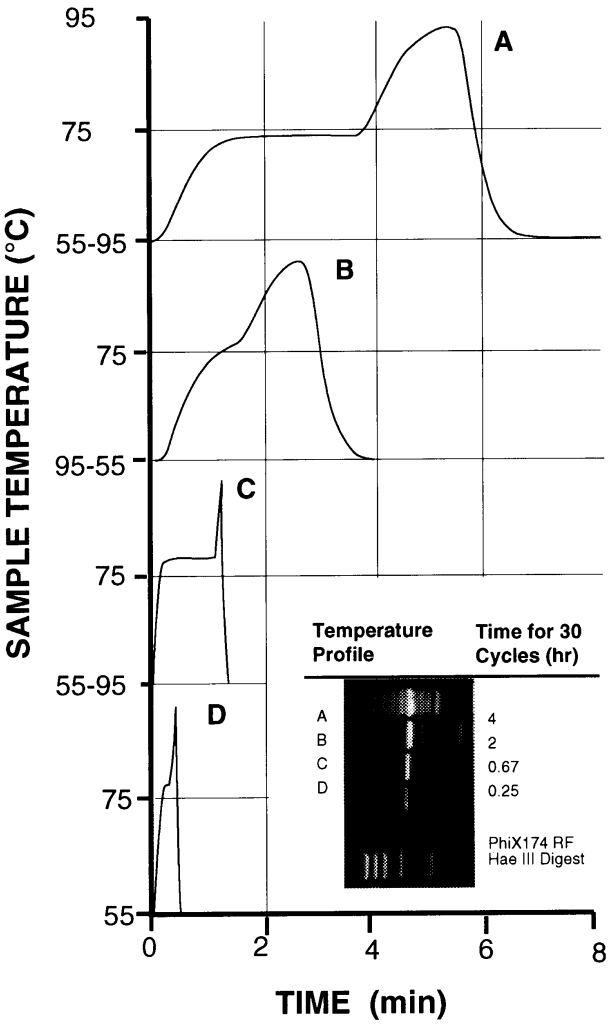

Figure 7. Effect of sample temperature-time profiles on product specificity. Samples were cycled 30 times through profiles A, B, C or D and 10 μl of the product electrophoresed and viewed by ethidium bromide staining. Profiles A and B were obtained using a commercial heat block instrument (Perkin-Elmer Cetus Thermal Cycler) set to "Step Cycle" mode (fastest possible transition times). Mineral oil (60 μl) was used to overlay 100 μl samples contained in microfuge tubes as recommended by the manufacturer. Samples were placed in a center well (D-5) and sample temperature profiles determined with a miniature thermocouple probe. Profile A resulted when the instrument was programmed to denature at 93 C for 1 min, anneal at 55°C for 2 min, and elongate at 74°C for 3 min. Profile B was obtained by modifying the program to minimize sample times at each temperature (35 sec at 55 C, 45 sec at 77 C, and 35 sec at 93°C). The rapid air cycler was used for profiles C and D. In profile C a 1 min elongation time at 77° C was used. Profile D uses a 10 sec elongation time at 77°C and is the 30 sec base profile described in Materials and Methods.

neous. Homogeneity of sample temperature is better with small samples and with symmetric, high surface area-to-volume containers. Small, thin capillary tubes are ideal; microcentrifuge tubes are not. Capillary tubes can be sealed with a Bunsen burner in less time than it takes to overlay the sample with mineral oil and close a microcentrifuge tube. After amplification, the ends of the glass capillaries can be quickly scored with a file and snapped off easily with less risk of aerosolization and contamination than microcentrifuge tubes. The capillary tubes serve both as a

transfer pipette and a container for temperature cycling. The amplified sample, already containing Ficoll and an electrophoresis indicator dye, can be directly emptied into a gel well without exposure to an intermediate pipette tip or to extraction procedures.

A common protocol for DNA amplification is 1 min at 94° C for denaturation, 2 min at 55° C for annealing, and 3 min at 72° C for elongation (16). If instantaneous temperature transitions were possible, one cycle would take 6 min. However, in conventional heat block machines it takes perhaps an additional 2 min to change the block temperature during each cycle (Figure 7A). When both heat block temperature transitions and sample time lags are considered, about 4 out of 8 min in each cycle, or 50% of the time is spent changing the sample temperature (Figure 7A). When rapid cycling or "turbo polymerase chain reaction" is attempted in conventional machines, the sample may be in continuous temperature transition (Figure 7B). It is understandably difficult to optimize the time/temperature settings for the three stages of DNA amplification when the sample temperature is always changing.

Recently, there has been a trend toward faster protocols for DNA amplification (5,15). Denaturation and annealing are claimed to occur "almost instantaneously" or "within a few seconds" once the appropriate temperatures have been reached by the sample (5,12,15). Adequate denaturation does appear to occur in less than 1 s (Figure 2) as long as the DNA is denatured by boiling before amplification is begun. Thoroughly boiling the template DNA before amplification is apparently necessary when very short denaturation times are used during cycling.

An annealing time of less than 1 sec was also found optimal (Figure 4). Kinetic studies on DNA renaturation predict rapid annealing because of the high primer concentration used in DNA amplification (18,19). Product yield and specificity were improved with shorter annealing times and faster denaturation to annealing transitions. Current commercial machines are limited to temperature transitions of less than 1°C/s for a total transition time (90°-55°C) of at least 35 s. In addition, most protocols call for an annealing time of 20-120 s According to Figure 4, poor specificity would be expected under these conditions. Experimentally, relatively poor specificity was seen when slow heat block amplifications (Figure 7A and 7B) were compared to rapid cycling amplifications (Figure 7C and 7D).

Although short denaturation and annealing times appear desirable, decreasing the elongation time can limit product yield. Primer extension is not instantaneous; Taq polymerase has an extension rate of 35-100 nucleotides/s at 72°C (5,6). As elongation times are decreased, product yields are eventually compromised (Figure 6). For the RS42/KM29 primer pair, a 10-s elongation time (total

amplification time of 15 min for 30 cycles) gives a moderately strong, specific band (Figures 6 and 7). If desired, the elongation time can be further reduced while maintaining product yield by increasing the concentration of polymerase (21). However, if maximal yield is more important than rapid amplification, total amplification times of less than 15 min will seldom be of practical value.

The amplification yield was greatest at an elongation temperature of 75°-79°C (Figure 5). This is a higher elongation temperature than conventionally employed, but is nearer the reported temperature optimum for the enzyme (2). Surprisingly, some 536-base pair product was detected even with an elongation time of 2.5 s at 75°-79°C (Figure 6). Elongation rates in DNA amplification may be higher at 75°-79°C than at 72°C and some elongation is expected to occur during temperature transitions.

The optimal annealing temperature for DNA amplification depends on the base content, nucleotide sequence and length of the primers and is related to the primer TM (5,12). Equations are available for estimating oligonucleotide TMs (7). However, in DNA amplification adjustments for lower monovalent cation concentration (17) and higher [Mg++] (3) are necessary. In our buffer system (20 mM KCl and 3 mM MgCl2) with the RS42/KM29 primer pair, the optimal denaturation temperature was around 92°-94°C (Figure 1), and an annealing temperature of about 55°C resulted in maximal specificity and yield (Figure 3). The propensity to anneal can most accurately be described by nearest-neighbor base-stacking interactions (1). The free energy released on heteroduplex formation should be related to the required annealing temperature in DNA amplification. The "mean stacking temperature" of an oligo has been correlated with the temperature at which 50% hybridization occurs (9). This "T50-hyb" for the RS42 and KM29 primers averages 59°C and 51°C for the two buffers investigated (9), close to the optimal 55°C annealing temperature found for DNA amplification in our system.

The amplification volume can be scaled up as desired to 25, 50 or 100 μl. Flexible silica capillary tubing as currently used in capillary electrophoresis can be coiled to provide a wide range of volumes with excellent heat transfer characteristics. A less expensive alternative is to use rigid glass capillaries of larger diameter. Temperature transitions and total amplification times are somewhat longer with larger diameter tubes. For example, while a 10-μl sample can be amplified in a 0.52-mm-i.d. tube in 15 min (base profile described in Materials and Methods), a 20-40-μl sample takes 20 min in a 0.96-mm tube, and a 50-100-μl sample takes 25 min in a 1.26-mm tube.

The choice of buffer and reactant concentrations in Figure 7 were optimized for rapid cycling. Other buffer systems may give a single specific band with con-

ventional slower cycling. For instance, the rapid cycling buffer included 500 μM dNTPs rather than 200 μM dNTPs which may decrease expected specificity. Nevertheless, using identical reactant concentrations (the same "master mix"), the relative specificity of rapid cycling was surprisingly superior to slower cycling.

Other modification in the buffer used deserve brief comment. Bovine serum albumin was required for amplification in capillary tubes. No amplification was obtained with gelatin, perhaps because of surface denaturation of the polymerase on the large surface area of the tube. Inclusion of Ficoll and an indicator dye in the amplification mixture is convenient and to our knowledge has not been previously reported. Xylene cyanole or tartrazine can be used as dyes, but bromphenol blue strongly inhibits the amplification reaction.

Increased specificity of DNA amplification by rapid cycling should be useful in sequencing, mutation detection and infectious disease diagnosis. With improved specificity, simple agarose gel electrophoresis may be sufficient for diagnosis in many cases without use of a probe internal to the primers. However, high specificity is not always desirable. Relatively low specificity is required when consensus primers are used to detect a group of related or rapidly mutating sequences or when the sequence to be amplified is not precisely known. Therefore, rapid cycling may be less suitable than slower, conventional cycling when primers have one or more mismatches with the template and equal amplification is desired. Conversely, better discrimination of mismatches should be attainable with rapid cycling.

This study was based entirely on a single primer pair for DNA amplification . However, we have also used rapid cycling to amplify the DNA defined by 20 different primer pairs from 6 different genes. The GC content of the primers varied from 23%-90% and the product lengths ranged from 80-1400 base pairs. The increased temperature/time definition of rapid cycling may allow rigorous correlation of the free energy released by nearest-neighbor base-stacking interactions with optimal annealing temperatures. This could be incorporated into an expert system for DNA amplification that suggests time, temperature and reaction conditions for any given primer pair.

Unfortunately, we are not aware of any rapid temperature cyclers that are commercially available. The apparent advantages of rapid cycling include decreased amplification times, increased specificity and decreased reagent cost because of smaller reaction volumes. Until the biomedical community and commercial manufacturers realize the advantages of rapid cycling, the technique will only be available to those willing to build, calibrate and optimize their own machines.

ACKNOWLEDGMENTS

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The RAPIDCYCLIST Newsletter

Volume 1 , Number 1, Spring 1992 **Pg. 77**

Optimizing RapidCycler™ DNA Amplification Reactions
Buffers and Reaction Components for RapidCycling
A R.A.P.I.D. Protocol for the ATC
Research in Progress at Idaho Technology

Volume 2 , Number 1, Spring 1994 **Pg. 91**

Creating a DNA Probe, Thermal Cycling with Degenerate Primers
Superior Quantitation of Rare mRNA's Using Rapid Cycling
Rapid Cycle Amplification of VNTR Loci for Engraftment in
Bone Marrow Transplantation
New from Idaho Technology
Reaction Mixes and Buffer Recipes
Rapid Cycle DNA Amplification - The 10 Most Common Mistakes

Volume 3 , Number 1, Fall 1995 **Pg. 112**

Capillary Tube Handling with the Rapidcycler
Use of Thin Walled Microcentrifuge Tubes with the Rapidcycler
Direct Sequencing of Long PCR Products
Rapid PCR Fingerprinting of Bacterial Genomes with REP Primers in
Capillary Tubes Using the Air Thermo-Cycler
Comparison of PCR Cycler Machines for Rapid and Sensitive Detection of Pathogens
New from Idaho Technology

The RAPIDCYCLIST newsletter has been modified to best fit into this manual. All of the articles that were published in the original versions of the newsletter have been included. There were only three publications of The RAPIDCYCLIST.

The RAPIDCYCLIST
Volume 1 , Number 1 Spring 1992

Optimizing Rapid Cycle DNA Amplification Reactions

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The complete optimization of a DNA amplification reaction unfortunately requires some trial and error. Optimizing an amplification requires finding conditions such that:

1. the template melts at the denaturation temperature,
2. the primers pair with their complement at the annealing temperature, but not with non-specific sequences.
3. Temperature and time conditions are adequate for the complete extension of the product.

Failure to meet any of these conditions will cause failure of the amplification reaction. You may notice that two of these conditions involve DNA duplex stability, so it's not surprising that two of the most important variables in DNA amplification, annealing temperature and salt concentration, both affect DNA duplex stability.

What follows is a short discussion of each of the components of an amplification reaction and then an outline of a systematic optimization protocol. This protocol has allowed the successful amplification of both DNA and RNA (via cDNA) using many different primer pairs.

A. Components of an Amplification Reaction.

1. Primers

Primer selection can greatly influence amplification success. Sometimes there is little or no latitude in the selection of primer position, in which case the following discussion is moot. Since the amplification reaction is quite robust, the chances are good that any primer pair can be made to work. However, with forethought, the optimization time can be minimized.

Given flexibility in primer selection, an intelligent choice of primers can simplify the optimization process and maximize both product yield and specificity. There are several commercially available programs for selection of primer pairs and we have found them helpful. These programs can help you avoid cross hybridization with other parts of your sequence, internal primer complementarity and the like.

If you are picking primers by eye you should try to make them similar in length (20 - 30 nucleotides) and GC content (30 -70%) as balanced primers are easier to optimize. We have found that using longer primers (25 - 30 nt) and relatively GC rich primers (50-60%) increases product yield with rapid cycling. There are reports of primer dimer formation when the last two 3' bases are complementary, but they are seldom seen in rapid cycling reactions.

Primer selection and DNA sequence analysis programs will provide a Tm value or even an "annealing temperature" for a given primer sequence. All of these numbers should be viewed with healthy skepticism. Different programs can give Tm values for the same primer that differ by as much as 20° C. In fact, the actual Tm value of primers under DNA amplification conditions is controversial because of the unknown effects of buffer constituents and changing DNA concentrations. We use primers at a final concentration of 0.5 μM. We make a 10X 5 μM stock solution containing one or both primers.

2. Template DNA

DNA amplifications are normally done on one of two types of template, genomic DNA or plasmid DNA. We usually put about 104 to 105 copies of the ta get sequence into a 10 μl reaction. For human genomic DNA that is about 50 ng of DNA, for Escherichia coli genomic DNA it is about 50 pg and for plasmid DNA it is about 100 fg.

The template DNA should be denatured before the cycling reaction begins. We link a two minute hold at 94° to the beginning of the cycling program. Alternately, genomic DNA that has been denatured by boiling and then stored at -20° never reanneals.

3. Mg Concentrations

We use 3 different reaction buffers in our amplification reactions and they differ only in Mg2+ concentration. The low, medium and high Mg2+ buffers contribute 1.0 mM Mg2+, 2.0 mM Mg2+, and 3.0 mM Mg2+ to the final reaction respectively.

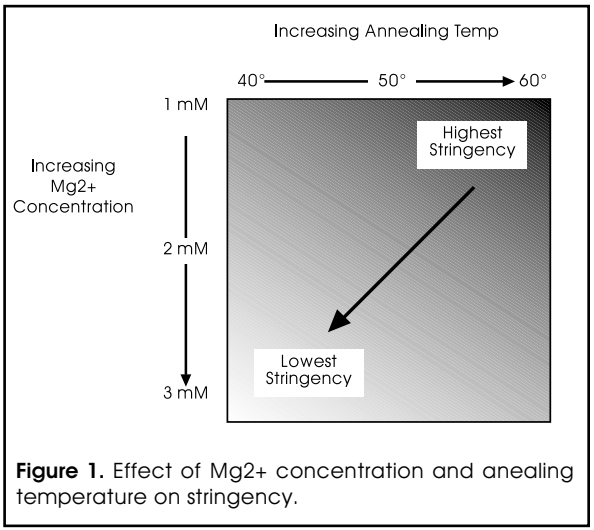

Figure 1. Effect of Mg2+ concentration and anealing temperature on stringency.

The optimal Mg2+ concentration is different for every template-primer set and must be determined experimentally. Magnesium ions stabilize DNA duplexes. Therefore lowering Mg2+ concentration increases stringency while raising Mg2+ concentration lowers stringency (Figure 1).

It has been reported that high Mg2+ leads to an increased rate of nucleotide misincorporation so if you are cloning your DNA products you may wish to avoid the high Mg2+ buffer.

4. dNTP's

Our usual final dNTP concentration is 200 μM of each dNTP. Increasing this concentration does not effect the yield of the reaction. If you are using low Mg2+ concentrations remember that each dNTP chelates a magnesium ion, so you need at least 0.8 mM Mg2+ to have any free Mg2+ at all.

5. Reaction Buffer

Our standard reaction buffer is a 10X buffer containing 500 mM Tris pH 8.3, 2.5 mg/ml crystalline BSA and MgCl2 at 10, 20, or 30 mM. The BSA is critical for preventing denaturation of the polymerase on the glass surface of the capillary.

If you plan to run your finished reaction on an agarose gel you can add 5% Ficoll 400 and 10 mM tartrazine to your 10X buffer. This allows you to add the reaction directly from the capillary tube to an agarose gel well. We use tartrazine instead of bromphenol blue or xylene cyanol because it does not affect the amplification reaction. Tartrazine runs faster than bromphenol blue.

If you wish, you may use commercially available reaction buffers for rapid cycling but you must add BSA. Failure to add BSA will cause denaturation of the polymerase and therefore failure of the reaction.

6. Enzymes

Most heat stable enzymes come at a concentration of 5 U/μl. We make a 1:12.5 dilution in a enzyme dilution buffer that consists of 10 mM Tris pH 8.3 and 2.5 mg/ml crystalline BSA. This gives a 10X stock solution. This dilute enzyme solution is stable for at least 2 days at 4°C. We have found little significant difference between the enzymes from different suppliers. However, different heat stable enzymes may differ in their reaction rates, and temperature and time paramters may need to be adjusted accordingly .

7. Reaction Volume

Our standard reaction volume is 10 μl. This produces enough DNA product for most applications. If you do need more DNA, multiple 10 μl capillaries can be filled from the same master mix or you can use larger 25 or 50 μl capillaries. The larger capillaries require a short (5-20 second) hold at denaturation and annealing to allow the larger sample to reach temperature. Because the temperature is not as well defined, we prefer to use multiple small capillaries.

8. Cycling Times and Temperatures

A cycling protocol requires setting three temperatures: denaturation, annealing, and elongation temperatures.

Denaturation should be set at as high a temperature as possible without killing the enzyme. We routinely use 94° C. Altering this temperature has not been helpful.

A rapid air cycler (Idaho Technology, Salt Lake City, Utah) can hold the denaturation time for as long as desired but we have not found any advantage in holding denaturation. We recommend a denaturation time of 0 seconds when using the standard 10 μl capillary tubes. Because of a larger thermal mass, samples in the 25 μl tubes require a hold time of 5-10 sec, and samples in 50 μl tubes require 10-20 sec.

We use 70° C as our standard elongation temperature. The extension rate vs. temperature curve (Figure 2) for Taq polymerase activity shows a broad

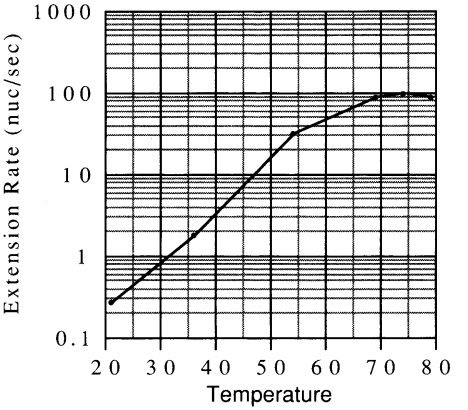

Figure 2. Extension rate vs temperature for Taq polymerase.

peak of about 100 nucleotides per second between 70 and 80° C.

The amount of time at elongation should be varied with product length. Taq polymerase catalyzes the addition of about 100 nucleotides per second at 70°C. For very small DNA products (target<100 bp) no elongation time at all is required. These products will elongate in transit. For medium length targets (100 - 500 bp) 5 to 15 seconds elongation is sufficient. Longer products must use proportionally longer times, approximately 15 and 30 seconds per kilobase of product.

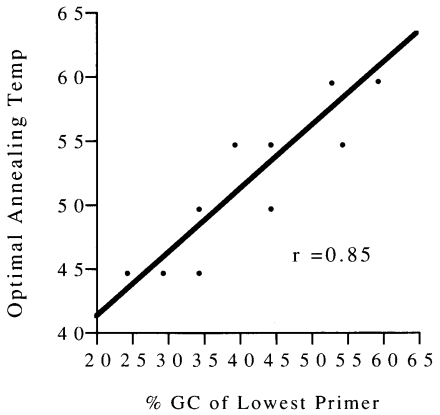

Figure 3. Correlation of optimum annealing temperature of GC content of lowest primer.

The annealing temperature is the most important variable in a DNA amplification. As mentioned above, calculated values of Tm should not be taken too seriously, but the consistent use of a single program can be helpful in predicting effective relative annealing temperatures for different primer sets.

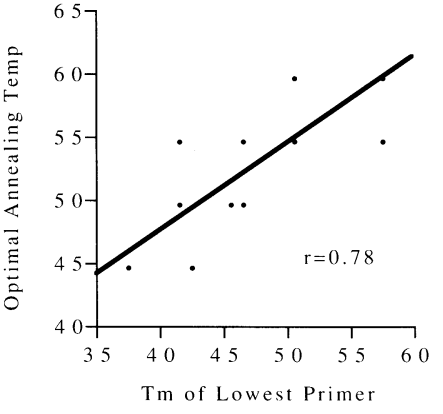

Figure 4. Correlation of optimum annealing temperature to Tm of lowest primer.

In a group of 15 pairs of twenty nucleotide long primers we correlated the percentage GC and the Tm calculated by a commercially available program to the final optimized annealing temperature. We found that the best predictor of annealing temperature was the GC percentage of the lowest GC primer (Figure 3). The Tm of the least stable primer was almost as good at predicting annealing temperatures (Figure 4).

The amount of time spent at annealing has a direct effect on the specificity of the amplification reaction (Figure 5). The longer you spend at the annealing temperature the more non-specific priming you see. You will notice in Figure 1

that the polymerase has significant activity at temperatures that are commonly used for annealing. As you spend more time at the annealing temperature there is a greater chance of non-specific priming and extension of undesirable product. We recommend that when using the standard 10 μl capillary tubes, you set your annealing time at 0 seconds to maximize specificity. As with the denaturation temperature, the 25 μl tubes require a hold time of 5-10 sec., and the 50 μl tubes require 10-20 sec. Some amplifications, especially those with low Tm's, may also require longer annealing times (5 to 15 sec).

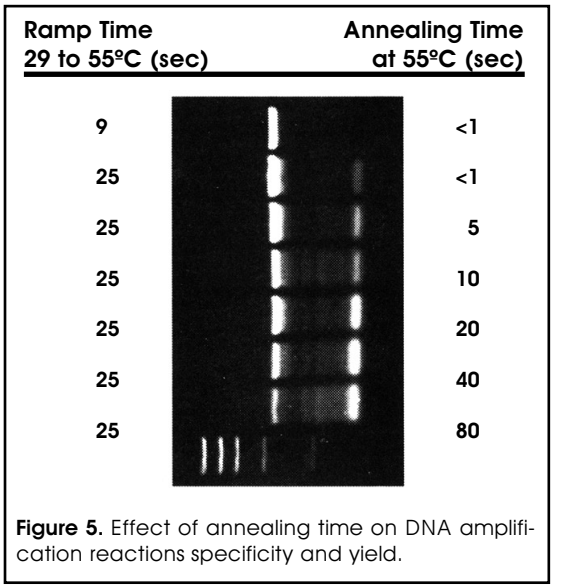

Figure 5. Effect of annealing time on DNA amplification reactions specificity and yield.

B. Systematic Optimization B. B. Protocol for Rapid Cycling

When trying to optimize a new primer pair we run test reactions at annealing temperatures of 40°, 50° and 60° C. At each of these temperatures we run high, medium and low Mg2+ concentrations (1.0, 2.0 and 3.0 mM MgCl2). This gives nine different reaction conditions which cover a wide range of DNA hybridization stringencies. The low Mg2+ buffer at 60° gives the highest stringency while the high Mg2+ buffer at 40° gives very low stringency (Figure 1). Usually one or more of these conditions will provide good specificity and yield. If needed, intermediate temperatures or Mg2+ concentrations can be tried in a second experiment.

If you are running a large number of primer pairs then nine reactions per pair can get a little out of hand. About 80% of primer pairs can be successfully amplified in the medium Mg2+ buffer at 40°, 50°, or 60°C. Even if none of these three conditions is ideal you will often get a clue as to what conditions to try next. If you have no band at 50° or 60° and a weak band at 40° then you will want to try the high Mg2+ buffer next. If 60° is giving non-specific amplification you will want to try the low Mg2+ buffer.

Conclusion

To paraphrase Robert Pirsig, "optimization of new primer pairs requires great peace of mind." Our lack of understanding of the amplification reaction prevents the formation of a set of rules for predicting conditions that will be successful. This lack of simple rules can make the optimization process frustrating. Fortunately there are usually only two variables to worry about, and the reasonable range of these variables is limited. Annealing temperatures are rarely less than 37° or more that 70°. Magnesium concentration is never less than about 1 mM and rarely more than 5 mM. It doesn't take many experiments to cover this range. With perseverance you can eventually get any primer pair to work.

Good Luck

Buffers and Reaction Components for Rapid Cycling

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Many different buffers and reactant concentrations have been reported for DNA amplification. Rapid cycle DNA amplification was originally optimized with the following buffer (1):

20 mM KCl
50 mM Tris, pH 8.5
3 mM MgCl2 (with 500 μM each dNTP)
500 ug/ml BSA

BSA is required when capillary tubes are used; gelatin is a poor substitute and greatly reduces amplification yield. Although we have tried conventional buffers that contain 50 mM KCl (2), a greater number of amplifications with various primers were successful at lower KCl concentrations. In sequencing reactions, the best extensions are reportedly obtained when no KCl is included (3). We now routinely use a buffer system without KCl:

50 mM Tris, pH 8.3
2 mM MgCl2 (with 200 μM each dNTP)
250 ug/ml BSA

Some primer pairs have only amplified with this "no KCl" buffer; conventional high KCl and our original buffer were not effective. Although it is probably true that no single buffer is best for all amplifications, we have successfully amplified about 80% of untested primer pairs with this buffer. Most of the remainder can be amplified by varying the Mg concentration from 1-3 mM.

DNA amplification reactions are very resilient, and many additives appear to have little or no effect on the reaction. Ficoll 400 (0.5 - 1%) and tartrazine (1 mM) are convenient to add to a reaction mixture before amplification if the products are going to be analyzed by gel electrophoresis. This allows direct transfer of the solution into a gel well from the capillary tube after amplification, without intermediate mixing (1). If you run many reactions and are looking for quick results, this is very convenient. By running parallel reactions with and without Ficoll/tartrazine, no significant differences in specificity or yield have been observed.

Whether certain buffers are more amenable to rapid vs conventional cycling has not been adequately studied. Reaction kinetics and equilibrium constants will change with different buffers, but the effects on amplification are poorly understood. Buffers other than those suggested here can be used for rapid cycle amplification, but BSA must be included in the reaction. It is convenient to add the BSA with the enzyme. A 10X enzyme solution of 0.4U/μl can be obtained from a 5U/μl enzyme stock by diluting in an "enzyme diluent" as follows:

11.5 μl enzyme diluent
(10 mM Tris, pH 8.3, 2.5 mg/ml BSA)
+ 1.0 μl enzyme

This is enough to run about 12 reactions. When the 10X enzyme solution is diluted, enough BSA is included for efficient amplification, even if no additional BSA is added with the buffer. The 50% glycerol storage media of most, enzyme preparations makes pipetting 1 μl very difficult. If accurate volumes are desired, microcapillary pipets (1 μl Microcaps, available from Sigma) can be used. The other components of a master mix can also be stored as 10X stocks. A 10X solution of human genomic DNA (50 μg/ml) conveniently has an absorbance of 1.0 at 260 nm. One μl of this 10X solution provides about 15,000 template copies per 10 μl reaction.

Table 1.

Rapid Cycle Reactant Concentrations

Component	[10X Stock]	[1X Reaction]	Volume/10µl
Buffer	500 mM Tris, pH 8.3	50 mM Tris	1 µl
	2.5 mg/mL BSA	250 µg/mL BSA	
	5-10%Ficoll	0.5-1.0% Ficoll	
	10 mM Tartrazine	1 mM Tartrazine	
Low Mg	10 mM MgCl2	1 mM MgCl2	
Med Mg	20 mM MgCl2	2 mM MgCl2	
High Mg	30 mM MgCl2	3 mM MgCl2	
dNTPs	2 mM each dNTP	200 uM dNTP	1 µl
Left Primer	5 µM	0.5 µM	1 µl
Right Primer	5 µM	0.5 µM	1 µl
DNA			1 µl
Genomic DNA (mammalian)	50 ng/µL or A (260)=1.0	5ng/µL	
Bacterial DNA	5-50 pg/µl	0.5-5 pg/µl	
Plasmid DNA	0.1-1.0 pg/µl	10-100 fg/µl	
Diluted Enzyme	0.4 U/µL	0.4 U/10µL	1 µl
	2.5 mg/ml BSA	250 µg/ml	
dH2O			4 µl

Note: BSA is present in both the 10X buffer and the enzyme diluent for a final concentration in the reaction of 500 ug/ml.

We usually use a 5 μM 10X solution of each primer. The concentration of primer stocks should be determined spectrophotometrically at 260 nm. The extinction coefficient of an oligonucleotide is affected by base sequence and is best estimated by considering neighboring pairs (4). Commercially available computer programs, such as Oligo 4.0 (National Biosciences, Hamel, MN) automatically perform the calculation.

A summary of reactant concentrations is given in Table 1. Suggested volumes of reaction components for a master mix for 4, 8, 16, 10 μ l samples are listed for convenience in Table 2 (below). Kits supplying the components of this system (exclusive of primers, template DNA, and enzyme) are commercially available (Idaho Technology, Salt Lake City, Utah).

Table 2

Component(10X)	Number of 10μl Reaction Tubes		
	4	8	16
Buffer	5 μl	9 μl	17 μl
dNTP's	5 μl	9 μl	17 μl
Left Primer	5 μl	9 μl	17 μl
Right Primer	5 μl	9 μl	17 μl
Template DNA	5 μl	9 μl	17 μl
Taq (0.4 U/μl)	5 μl	9 μl	17 μl
dH2O	20 μl	36 μl	68 μl
Total Volume	50 μl	90 μl	170 μl

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A RAPD Protocol for the Air Thermo-Cycler

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Idaho Technology's Air Thermo-Cycler is unique among commercial thermal cyclers in the speed with which PCR reactions can be performed. The RAPD reaction, a PCR technique for generating useful genetic markers (Williams et. al. 1990. Nucleic Acids Research. 18: 6531-6535), also runs much faster in the ATC. Published RAPD protocols are based on the use of metal block machines and require relatively long total cycling times. The RAPD reaction can be economically performed in the Idaho Technology ATC with a total cycling time of less than 90 minutes.

Template DNA should be clean and relatively free of RNA. Treatment with RNase followed by an alcohol precipitation is sufficient to remove most of the RNA. Clean DNA may give good RAPD products even when it is significantly degraded. 5X solutions of template and primer are prepared in a TE buffer that is 1mM Tris (pH=7.5) and .1mM EDTA (pH=8.0). A 25X dNTP and MgCl2 solution is prepared in distilled H20. 5X reaction buffer contains 250 mM Tris (pH=8.5), 5mM MgCl2, 100 mM KCL, 2.5 mg/ml BSA, 12.5% Ficoll 400 and .1% xylene cyanole. For a set of 100 reactions a master mix containing dNTP's, MgCL2, Taq DNA polymerase (Promega Corp.), and reaction buffer is prepared by mixing 200 μl 5X

buffer, 40 µl 25X MgCl2- dNTP's, 12 µl Taq polymerase (5 units/µl), and 348 µl distilled H20. Reactions are then prepared in 10 µl volumes by combining master mix, 5X template, and 5X primer in the ratio 3:1:1. The final concentrations in volumes of 10 µl should be 2 ng/µl template DNA, .4 µM primer, 100 µM each dNTP, 2 mM MgCl2, .06 U/µl polymerase and 1X reaction buffer.

Amplification is divided into two steps. For the first two cycles the thermal profile is 1 minute at 92° C, 7 seconds at 42° C, and 70 seconds at 72° C. Subsequently, an additional 38 cycles are performed with denaturation for 1 second at 92°C, annealing at 42° C for 7 seconds, and elongation at 72° C for 70 seconds. Following these forty cycles the temperature should be held constant at 72° C for 4 minutes.

Some Comments on Reaction Optimization

A particular RAPD product generated from a unique primer and template combination will require a specific set of optimum conditions. Our goal was to get good bands for a large number of primers making our protocol as general as possible. Also, we wanted to be able to run the reactions in a short amount of time to maximize throughput. Interactions between concentrations, times, and temperatures are important. Changing the value of any parameter may change some reaction results. However, reaction parameters near those given here will give good results. For example, annealing for 7 instead of 8 seconds is somewhat arbitrary. Attempts to improve the efficiency of the reaction by significantly lowering or raising the polymerase concentration has not worked, in general. Using the protocol described above, we have obtained satisfactory reaction products from thousands of RAPD reactions.

Results

The following figures show some results from our lab using the above protocol.

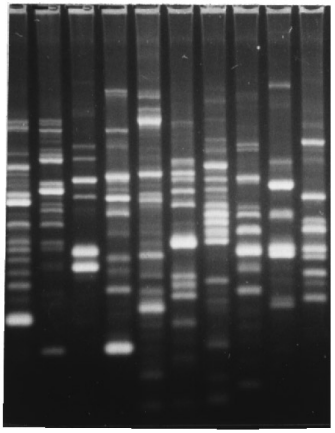

Figure 1. The gel shows RAPD amplification products from 10 different primers with a single Phaseolus vulgaris (bean) DNA preparation. The number of bands amplified for a given primer can vary from 1 to about 16.

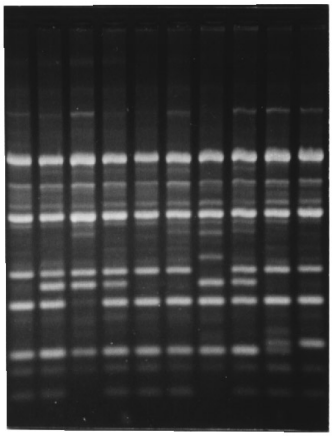

Figure 2. The gel compares 10 different P. vulgaris genotypes amplified with a single primer.

Research In Progress at Idaho Technology

Kirk M. Ririe
Idaho Technology

Idaho Technology is committed to improve the state-of-the-art in rapid cycling, instrumentation and accessories. Several recent developments are worth noting.

Linear Actuator Tests:

We recently tested a prototype instrument outfitted with a linear actuator in place of the solenoid on the 1605 ATC. Our primary objectives are to eliminate the noise produced by the solenoid and to gain better control of the temperature/ time curve. While we have done our best to make the 1605 ATC a flexible instrument, there are some restrictions imposed by the original design. Since the solenoid is either fully open or fully closed, the machine is limited to a single cool down rate. This rate is factory set by adjusting the solenoid to cool down from 94° C to 55° C in about 8 sec. Cool down rates as low as three secs are possible with this design, however, product yield is decreased with rapid cooling. This is due to the temperature rebound which occurs when the door closes. Apparently, there

is not enough time for the primers to anneal and the enzyme to function before the increasing temperature causes denaturation. A linear actuator can in theory produce cool down rates in the range of 3-4 secs and then hold the lower temperature for precisely the required time. This would allow a slight decrease in cycle time, yet substantially increase product yield. This work is especially important for low annealing temperature protocols such as RAPD.

We are committed to developing a more flexible instrument, while retaining the simplicity of the ATC. The linear actuator will allow much finer control of the temperature/time curve. Two temperature cycling and three temperature cycles with unusually high annealing temperatures will be facilitated. Upgrades to a linear actuator system should be available by the summer of 1992.

Sample Handling Advances

We recently received sample quantities of a 25 μ l plastic capillary tube. Initial tests confirm that the reaction runs at slightly reduced speeds compared to glass capillary tubes. Using the 25 μ l plastic capillary tubes, the sample comes to temperature in 5-10 secs, which is comparable to sample response when using 25 μ l glass capillary tubes. We intend to continue our tests and make a positive displacement sample handling system available soon.

We have recently tested a microscope slide rack using 24 x 60 mm cover slips (Fisher Scientific). Early test results look promising. The slide rack will be available for purchase in May.

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Creating a DNA Probe,Thermal Cycling with Degenerate Primers

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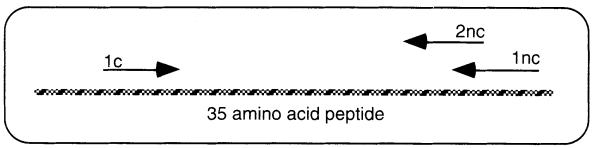
We are cloning the DNA from a structural protein in Xenopus leavis to further characterize it. A DNA probe was needed for Southerns, northerns and probing libraries for our gene of interest. The following is our procedure using the Air Thermo-Cycler to clone and amplify a fragment of DNA using degenerate primers. We found increased primer concentration as well as longer annealing times were beneficial in obtaining DNA products from degenerate primers.

Primer Design

The protein of interest was digested with endo-Asp-N to obtain protein fragments for amino acid sequencing. Of these, a 35 amino acid peptide was chosen to design degenerate primers for amplification of the peptide DNA. Coding (1c) and non-coding (1nc) primers were made from terminally located amino acids with minimal codon degeneracy. A third non-coding (2nc) primer was made internal to 1nc primer (see figure).



Each primer was 26 nucleotides long. All combinations of nucleotides at codon wobble positions were synthesized with the following exceptions: inosines were used for 4-fold degeneracy at the wobble position when appropriate

(according to Molecular Cloning, a Laboratory Manual. Sambrook et al., page 11.18); to accommodate a serine in 1c and a leucine in 1nc, primers had to be made in duplicate; for serine, the codons TCI and AGT/C were used; for leucine (1nc, 2nc), IAG and T/CAA were used. Each primer was synthesized with a GGC clamp and an EcoR1 site at the 5 ? terminus. The degeneracy of the 1c, 1nc, and 2nc primers were respectively 48 fold, 8 fold and 48 fold. The expected size of the product from the 1c and 1nc primers was 100 bp, and from the 1c and 2nc primers, 94 bp.

Reaction Mix

primers	7 μM working concentration; 1 μl each of 1c and 1nc
template	7.6 ng/μl Xenopus leavis oocyte cDNA; 1μl
[Mg2+]	30 mM; 1 μl
dNTP mixture	2 mM each dNTP; 1 μl
10 X buffer	500 mM tris pH 8.3, 2.5 mg/ ml BSA, 1 μl
enzyme	1 μl Taq polymerase diluted 1:12.5 in enzyme dilution buffer (10 mM tris pH 8.3, 2.5 mg/ml BSA)
water	to 10 μl total volume

Thermal Cycling Conditions

These conditions produced the expected 100 bp fragment in small amounts as visualized on a 4% Nusieve low melting temperature agarose gel. The band was cut from the gel (approximately 2X2X4 mm chunk) and used as a template in subsequent thermocycling reactions.

Each 10 ul reaction was done in heat sealed glass capillaries.

Initial hold -- 2 minutes 94°C

2 cycles	D: 94°C 0 sec,	A: 40°C 7 sec,	E: 74°C 5 sec
5 cycles	D: 94°C 0 sec,	A: 42°C 7 sec,	E: 74°C 5 sec
23 cycles	D: 94°C 0 sec,	A: 45°C 7 sec,	E: 74°C 5 sec

Confirmation of the 100 bp product

To confirm the accuracy of our 100 bp product, we attempted to amplify a smaller fragment using the 100 bp cycling product as a template with the internal non-coding primer (2nc) and the original coding primer (1c).

The 100 bp product isolated in agarose was heated at 100 °C until melted, and 500 µl TE was added. Two µl of this mixture were used as the template in a 10 µl reaction. One µl each of the 1c and 2nc primers was used, and the other parameters were as described above.

The thermal cycling reaction was run with an initial 2 minute denaturation at 94 ° C followed by 30 cycles: 0 sec at 94 °C (denaturation), 12 sec at 50 °C (annealing), 5 sec at 74 °C (elongation).

This reaction produced a 94 bp band as seen on a 4% Nusieve agarose gel indicating that the 100 bp fragment was the correct DNA sequence. A duplicate reaction to the one directly above was done with the 100 bp fragment as template and the original outside primers (1c and 1nc) to amplify the 100 bp fragment. One 10 µl reaction gave approximately 30 ng of product.

The 100 bp product was cloned directly into the pCRII® vector from the Invitrogen TA Cloning Kit. Subsequent DNA sequencing of this vector confirmed that this product coded for the original amino acid sequence and will be used as a probe for subsequent experiments.

Superior Quantitation of Rare mRNA's Using Rapid Cycling

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After a long period of skepticism, quantitative PCR is finally gaining acceptance in the molecular biology community. No one doubted that PCR could be quantitative in theory, but there was a general consensus that the efficiency of DNA amplification would be too sensitive to interference for practical quantitation. Small effects on the reaction's efficiency, it was argued, would destroy the quantitative value of PCR. Quantitation of mRNA added the additional complication of the reverse transcription step.

Despite these initial concerns, it has now been thoroughly demonstrated that the quantitative power of reverse transcriptase PCR (RT-PCR) is as good or better than the traditional methods of mRNA quantitation such as northern blot (1), dot blot (2), and in situ hybridization (3). Two recent papers from the John Weis lab report a sensitive RT-PCR assay using rapid air thermocycling (4,5). The Weis lab was trying to measure mRNA for the complement receptor Cr2, a rare mRNA in mouse spleens. They were unable to quantitate the message when they used slow heat block cyclers because of very low yields of product DNA and highly variable amounts of product. They switched to an Air Thermo-Cycler and solved both of these problems. The amount of DNA produced was at least 100 fold greater in the air cycler than in the heat block instrument and the variability problem disappeared (Figure 1).

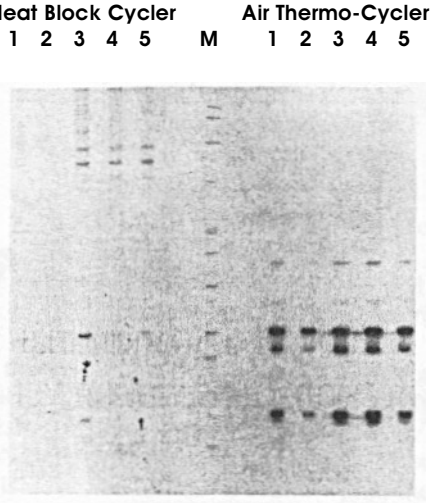

Figure 1. Comparison of RT-PCR products using heat block instrument and the Air Thermo-Cycler (ATC). Five different spleen cDNA samples were set up for PCR amplification and equally split between the standard heat block instrument (first 5 lanes) and the ATC (last 5 lanes) for the same number of cycles. Quantitation of these results (cutting the bands out of the gel and counting incorporated 32P-dCTP) indicated that there was at least 100 fold more product from the ATC than the heat block machine.

This short review will include some general considerations in quantitative PCR followed by the detailed Weis protocol.

The Linearity Problem

The amount of DNA produced in a PCR reaction is predicted by the well known equation:

$$y = x(E)^n$$

where **y** is the concentration of DNA produced by the amplification
x is the initial concentration of DNA
E is the efficiency of the reaction. For example, in a reaction where the amount of DNA is doubled every cycle, the efficiency is 2.
n is the number of amplification cycles

This equation can be linearized to:

$$\log(\mathbf{y}) = \mathbf{n}\log(\mathbf{E}) + \log(\mathbf{x})$$

The **y** intercept of this line gives the log of the starting concentration of DNA while the slope of the line gives the log of the efficiency of the reaction.

When the DNA concentration of an amplification is determined after varying numbers of cycles, the results fit quite nicely to the equation above during the early cycles. Efficiency is reduced during the later cycles of an amplification reaction (Figure 2). This is probably due to primers competing less effectively with template reannealing and a lower molar ratio of enzyme to product. The number of cycles after which these effects become important depends on the initial concentration of DNA.

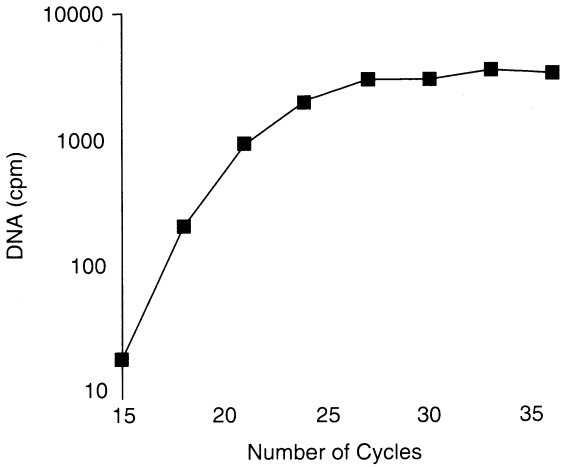

Figure 2. Amplification of mouse splenic cDNA with primers complimentary to the complement receptor Cr2. Eight identical samples were prepared with 250 ng of cDNA and removed sequentially every third cycle. After electrophoresis and autoradiography, bands were excised and quantitated by liquid scintillation counting (from Weis 1991).

When doing a quantitation experiment with the Air Thermo-Cycler, a typical experiment would include making up a large master mix, filling multiple capillaries from that master mix, and starting all the tubes at the same time. As the reaction goes on, tubes are pulled out at increasing numbers of cycles. The amount of DNA in each tube can be quantitated in various ways. The points that fall in the log-linear portion of the curve can be used to determine the amount of starting material and the efficiency of the reaction. For Figure 2, the efficiency of the reaction during the log-linear phase was about 1.7, which is typical for a real reaction.

The Quantitation and Detection Problem

The most common technique for detection and quantitation of DNA is radiolabeling with 32P. Amplified products can be labeled by incorporation of radiolabeled nucleotides or by end labeling one of the primers. End labeling tends to be more sensitive because a higher fraction of the product carries a label (6) , but labeling by incorporation is easier if you don't need the sensitivity. After the amplification, reactions are size separated by gel electrophoresis. The gels can then be directly quantitated by autoradiography using film or a PhosphorImager type system . The limited linear range of film (usually 3 orders of magnitude or less) makes this approach difficult. Phosphor Imager type systems are convenient and have extended linear ranges (5 to 9 orders of magnitude) but are very expensive. The Weis protocol uses labeling by incorporation of 32P-dCTP, location of the product by autoradiography, and quantitation by excision of the band and liquid scintillation counting.

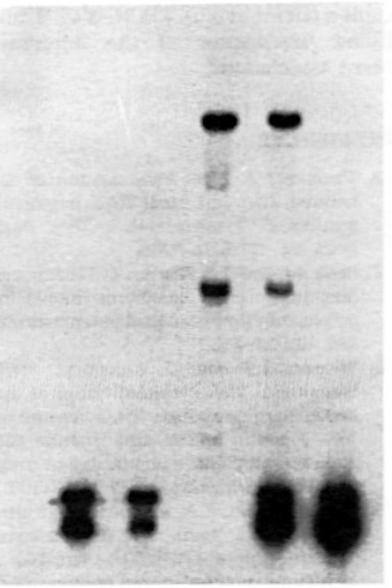

Figure 3. Effect of amplifying two different gene products in one reaction. PCR analysis of 100 ng of splenic cDNA with Cr2 and ß-actin oligos. Lanes 1-3 were done for 18 cycles. Lane 1, Cr2 oligos alone; lane 2, ß-actin oligos alone; lane 3, Cr2 + ß-actin oligos. Lanes 4-6 were done for 24 cycles. Lane 4, Cr2 oligos alone; lane 5, Cr2 + ß-actin oligos; lane 6, ß-actin oligos alone (from Tan 1992).

Some users of the Air Thermo-Cycler are hesitant to load glass capillaries with a radioactive reaction mixture

because of a fear of breakage. While Weis reports that this has not been a problem, plastic capillary tubes are now available (see "New from Idaho Technology" in this issue).

The Reverse Transcriptase Problem

In most protocols the reverse transcription is primed with the same primer that is later used for the amplification. The Weis group uses random hexamers to prime the cDNA synthesis and they report several advantages to this approach. First, it ensures that all RNA's are represented equally in the cDNA pool. Second, as reverse transcription is done at low temperatures, using 20-mers to 30-mers can lead to synthesis of cDNA's from non-specifically hybridized primers. These products might be specifically amplified during the quantitation.

The Relative versus Absolute Quantitation Problem

When measuring product by radiolabel, it is difficult to convert CPM's to absolute measures of DNA quantity. One solution to this problem is to set up an external standard curve by running known amounts of DNA each in their own reaction tube. Unfortunately this straightforward method has run into trouble due to large variation in the efficiency of different reactions. Further complications arise with RT-PCR because of the desire to control for the efficiency of the reverse transcriptase step. These problems have led to the use of internal standards of various types (7).

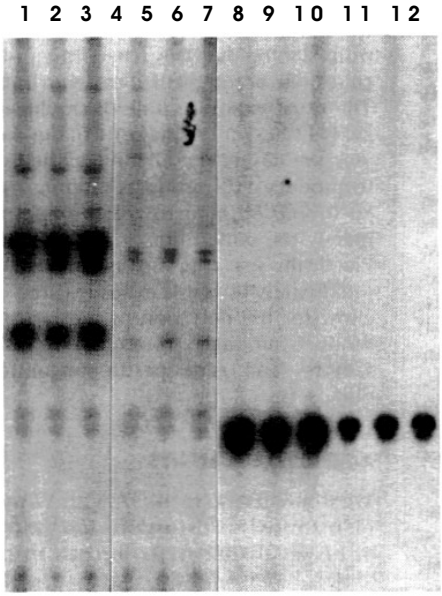

Figure 4. Reproducibility of PCR amplification for quantitation of products: multiple tissue samples. PCR analysis for 24 cycles with Cr2 oligos (lanes 1-6) and ß-actin oligos (lanes 7-12) with 100 ng of cDNA generated from three different spleens (lanes 1-3 and 7-9) and livers (lanes 4-6 and 10-12) (from Tan 1992).

The simplest internal standard is to simultaneously quantitate the sequence of interest with some more or less invariant "housekeeping" mRNA. If the level of the housekeeping gene's message is constant

between samples, then the amount of the unknown transcript can be reported in relative terms. Popular genes for standardization are ß-actin and HLA genes. All of these internal standard methods are based on the presumptions that: 1) the reverse transcription is not biased between the standard and test transcripts, 2) the amplification of the standard and the unknown occur with the same efficiency, and 3) the amplifications do not interfere with each other significantly.

Weis uses ß-actin mRNA as an internal standard (Figure 3). The autoradiograph shown in figure 3 shows that both products can be simultaneously amplified with minimal interference.

The Variability Problem

Sample-to-sample variability has long been a problem with RT-PCR. The efficiency of reverse transcription has been reported to vary from 5% to 90% (8), while the amplification itself may vary up to 200-300% between duplicate reactions. The Weis group reports good reproducibility not only between duplicate aliquots of the same cDNA but also between tissue samples (Figure 4).

The Protocol

1.Total RNA was prepared by the method of Chirgwin et al. (9)

2.RNA (5 μgs) was reverse transcribed in 1X RT buffer(GIBCO-BRL), 0.125 mM each dNTP, 0.5 μg random hexamers (New England Biolabs) and 400 units of Moloney virus reverse transcriptase (GIBCO-BRL) in a 50 μl reaction. The reaction was incubated at 37°C for 60 minutes. DNase free RNase was added and incubated for 5 minutes at 37°C. The reaction volume was adjusted to 270 μl with 0.4 M NaCl and was phenol extracted and precipitated with ethanol. cDNA concentration was determined by UV absorbance.

3.The optimal cDNA concentration and number of cycles was determined by a titration from 1 to 500 ng of cDNA and from 18 to 39 cycles. Optimal parameters were 200 ng of cDNA for 20 cycles. Each 10 μl reaction contained 200 ng of cDNA, 70 pmoles of each primer, 50 mM tris pH 8.3, 3 mM MgCl2, 20 mM KCl, 0.5 mg/ml BSA, 0.2 mM each dNTP, 2.5 uCi [32P]dCTP(3000 Ci/mmol; New England Nuclear), 0.72 units AmpliTaq DNA Polymerase (Cetus). To improve reproducibility, a master mix was prepared without primers and then aliquoted to separate tubes containing the different primer pairs. These mixtures were then aliquoted to the cDNA samples. Each 10 μl reaction was loaded into a glass microcapillary tube (Idaho Technology) and the ends were flame sealed. Capillaries were cycled in the 1605 Air Thermo-Cycler (Idaho Technology). Cycling parameters

were denaturation, 94°C for 1 sec; annealing, 59°C for 1 sec; elongation, 72°C for 4 seconds (products ranged in size from 80 to 200 base pairs). Total cycle time was 24 seconds.

4.Following amplification the ends of the capillary tubes were scored and the samples removed using a microaspirator and then 5 μl were electrophoresed in a 6% acrylamide gel. Radioactive bands were detected by autoradiography and then the bands were cut from the gel for quantitation by liquid scintillation counting. A 32P-end labeled MspI digest of pBR322 was used as a size standard.

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Rapid Cycle Amplification of VNTR Loci for Engraftment in Bone Marrow Transplantation.

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Bone marrow transplantation is now standard therapy for a range of diseases including many hematologic malignancies, some solid tumors, and some acquired or inherited hematologic and immunologic diseases. Many of these disorders result from a malfunctioning bone marrow, and the only cure is to inactivate the diseased bone marrow and replace it with healthy marrow. After the original marrow is destroyed, healthy marrow from a donor is infused into the recipient. Bone marrow transplantation may be: 1) autologous (where healthy stem cells have been previously harvested from the same individual), 2) syngeneic (where the donor is an identical twin), and 3) allogeneic (where the donor is different genetically from the recipient). In allogeneic transplantation, it is possible to determine the success of transplantation by monitoring the genotype of cells appearing in the peripheral blood. If the recipient type converts to the donor type, successful engraftment has occurred.

Variable number of tandem repeat (VNTR) loci are regions in the human genome where a short nucleotide sequence is repeated in tandem for a variable number of times. If flanking primers are placed outside of the repeats, the number of tandem sequences in any particular allele determine the length of the amplified product. Some VNTR loci are highly polymorphic with over 10 different alleles and are very useful for establishing individuality by genotype. For highly polymorphic loci, homozygosity is uncommon and two bands are expected at each locus because of the diploid nature of human cells. VNTR loci are commonly used in forensics to establish identity and can also be used to establish donor vs. recipient type in peripheral blood leukocytes after bone marrow transplantation. Since peripheral blood leukocytes originate in the bone marrow, the type of circulating leukocytes establishes the type of hematopoietic cells populating the

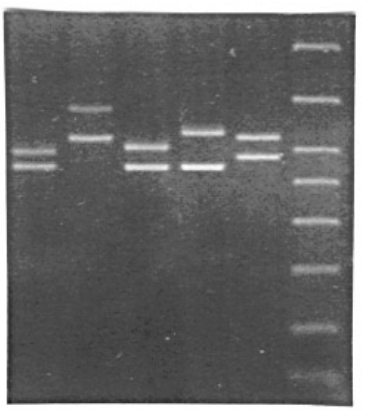

Figure 1. DNA samples from five unrelated individuals amplified with primers for the D1S80 locus2.

bone marrow. An example of DNA amplification of a VNTR locus in 5 unrelated individuals is shown in Figure 1.

Siblings are often used as donor/recipient pairs in bone marrow transplantation because they may match at HLA loci and have fewer problems with graft/host acceptance. HLA and VNTR loci are not linked and follow classical Mendelian inheritance. If siblings are matched at HLA loci for transplantation, they have a one in four chance of receiving the same parental VNTR alleles at any particular locus. If they do receive the same VNTR alleles at one locus, that particular locus is not useful for distinguishing donor vs. recipient type. However, most of the time, siblings will differ by either one allele (50% of the time) or two alleles (25% of the time). DNA from peripheral blood leukocytes needs to be isolated from donor and recipient before bone marrow transplantation, so that informative VNTR loci can be identified. Since there are many VNTR loci, finding differences between recipient and donor is not difficult, even for siblings. In the case of syngeneic or autologous transplantation, genotyping studies are not informative. The VNTR loci used here are HGM locus D17S301 and D1S802. All PCR reactions were run with standard rapid cycling techniques3-5 in an Idaho Technology 1605 air cycler with buffers and reagents supplied by Idaho Technology (1761 Optimizer Kit). The Mg2+ concentration was 2.0 mM. Cycling parameters were denaturation at 94° C for 0 sec, annealing at 55° C for 0 sec, and elongation at 73° C for 20 sec for 30 cycles. The total cycle time was 23.7 min. The samples were loaded directly on a 1.5% Agarose gel and electrophoresed at 5 V/cm.

VNTR Amplification in Bone Marrow Transplantation

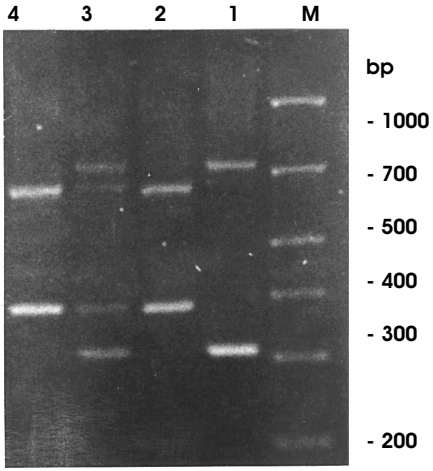

Figure 2. Bone marrow transplant engraftment by 1 month.
The VNTR locus HGM D17S301 was used.
Lane 1: Recipient before transplantation.
Lane 2: Donor.
Lane 3: Recipient 2 weeks after transplantation.
Lane 4: Recipient 4 weeks after transplantation.
In lane 3 the patient shows bands from both the recipient and the donor. In lane 4 the patient shows only donor bands suggesting successful engraftment.

Figure 2 illustrates a typical example of engraftment. This is a sibling transplant where all four alleles are different. At 14 days after transplantation, both donor

and recipient bands were observed. Residual recipient lymphocytes may circulate for 2-3 weeks after transplantation. However, recipient bands should disappear by 4 weeks if engraftment has occurred.

Figure 3 illustrates a typical example of disease recurrence after bone marrow transplantation. This is a sibling transplant where one allele is shared between donor and recipient types. At 36 days post bone marrow transplantation, both donor-specific and recipient-specific alleles are apparent. This indicates that the donor marrow has not entirely supplanted the recipient marrow at 36 days. At 100 days post bone marrow transplantation, only the recipient bands are present, indicating failure of engraftment and recurrence of disease.

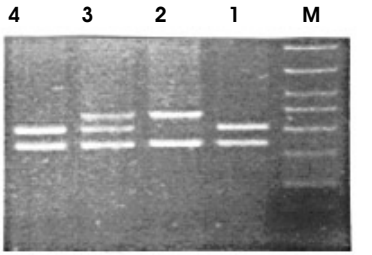

Figure 3. Disease recurrence after bone marrow transplantation. The VNTR locus HGM D1S802 was used.
1: Recipient before transplantation.
2: Donor.
3: 36 days after bone marrow transplantation.
4: 100 days after bone marrow transplantation.
In lane 3, alleles from both the donor and recipient are present at approximately equal amounts. After 100 days (lane 4), the unique donor band has disappeared and only the original recipient alleles are present.

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New From Idaho Technology

Kirk Ririe
Idaho Technology Inc.

Polycarbonate tubes

There has been a great deal of interest displayed by users of the 1605 Air Thermo-Cycler (ATC) in the possibility of using plastic capillary tubes to augment the glass capillary tubes currently standard in our instrument. The results of our tests on various plastic tubes have been very encouraging. Our selection for final testing is poly carbonate tubing which has thermal response characteristics almost identical to our 10 μl glass tubes. The polycarbonate does not interfere with the reaction and it should be of great help in those situations where the fragility of glass capillary tubes is an excessive hazard. However, those who are interested in using plastic capillary tubes should be aware that plastic tubes are not without their drawbacks.

Glass tubes can be easily loaded either singly or eight at a time by capillary action. However, hydrophobic plastic tubes require a loading mechanism such as a micro-aspirator or a similar device. We are working on ways of loading and sealing eight tubes at a time; but at present it can only be done one tube at a time.

The second drawback is sealing the ends of the plastic tubes. It is tricky but possible to flame seal plastic tubes by intentionally igniting the ends. For many people, this tends to be somewhat disconcerting; therefore, we have developed an electric tip sealer.

The last potential problem with plastic tubes is price. At a cost of approximately $80 per 1000, plastic tubes will be about twice as expensive as similar glass tubes. Even at that price, plastic tubes would still be less expensive than other second generation sample containers. We will know more about pricing after final testing on the tubes is complete.

We hope to be completely finished with the final tests on the plastic tubes and have the tubes and the sealers available in June of '94, barring major catastrophe. (10 μl tubes, part number 1714; tube sealer, part number 1740)

Modular Tops

In other hardware news, some of our earlier customers may be interested in a change made in the design of the 1605 cycler. The plastic top now has removable modules for loading and unloading tubes. The entire module is removable from the rest of the top to allow easier loading and unloading. Each module holds 16 tubes. To help ensure a good fit of all sizes of tube, modules are available in two sizes, 10 μl and 50 μl. An upgrade kit to a modular top is available from Idaho Technology, part number 1869.

Module Racks

A rack for holding the capillary tube modules is also now available. Each rack will hold three filled capillary tube modules. These module racks should help eliminate damage to capillary tubes when filled modules are set down prior to reinsertion into the instrument top. The part number is 1735.

Improved Buffer System

We have made several improvements to the buffers optimized for rapid cycling. Traditionally we have used Ficoll and tartrazine to increase the density of our buffer and make it visible for direct loading of product onto gels. We now recommend substituting sucrose for Ficoll, and cresol red for tartrazine.

For optimizations we have traditionally recommended using a three-by-three matrix of 3 mM, 2 mM, and 1 mM Mg2+ run at three annealing temperatures; 40°C, 50°C, and 60°C. However, our experience is that most reactions optimize at the higher end of the Mg2+ concentration, therefore we now recommend using 2 mM, 3 mM and 4 mM Mg2+ in the high, medium and low buffers.

We will include the new buffers free with all reagent orders for the next few months and if the reaction is positive, we will switch to the new system for individual buffer orders and the Optimizer Kit. As usual we are also publishing the reagent constituents in case you choose to make your own buffers. On the following pages are procedures for running individual reactions, making mastermixes, and making the reaction constituents themselves.

Reaction Mixes and Buffer Recipes

from Carl Wittwer's laboratory

Reaction Constituents for One 10 µl Reaction

Component	[10X]	[Reaction]	Separate	Combined
DNA (human genomic)	50 ng/µL or A 260 = 1.0	50 ng/10µl	1 µl	1 µl
Primers				
Separate				
Primer 1	5 µM	0.5 µM	1 µl	
Primer 2	5 µM	0.5 µM	1 µl	
or Combined				
Primer 1 + 2	5 µM each	0.5 µM each		1 µl
Nucleotides	2 mM each dNTP	200 µM each dNTP	1 µl	1 µl
Buffer	500 mM Tris, pH 8.3 2.5 mg/ml BSA 20% (w/v) Sucrose 1mM Cresol Red	50 mM Tris, pH 8.3 250 µg/ml BSA 2% (w/v) Sucrose 0.1 mM Cresol Red	1 µl	1 µl
Low Mg2+	20 mM MgCl2	2 mM MgCl2		
Medium Mg2+	30 mM MgCl2	3 mM MgCl2		
High Mg2+	40 mM MgCl2	4 mM MgCl2		
Enzyme	0.4 U/µL	0.4U/10µl	1 µl	1 µl
dH20/other			4 µl	5 µl

Amplification Procedure

1. Prepare master mix without DNA and without primers weekly:

For <50 runs at a 10 µl reaction volume:

Dilute Enzyme to 0.4 U/µl

11.5 parts Enzyme diluent
(10 mM Tris pH 8.3, 2.5 mg/ml BSA)
1 part Enzyme (5 U/µl)

For separate 5 uM primers:
4 parts dH20
1 part buffer
1 part 2 mM dNTPs
1 part 0.4U/µl Enzyme

For combined 5 uM primers:
5 parts dH20
1 part buffer
1 part 2 mM dNTPs
1 part 0.4U/µl Enzyme

Mix and store at 4°C for < 1 week.

For >50 runs at a 10 µl reaction volume:

For separate 5 uM primers:
308 (61.5 parts) dH20
63 µl (12.5 parts) buffer
63 µl (12.5 parts) 2 mM dNTPs
5 µl (1 part) 5U/µl Enzyme

For combined 5 uM primers:
370 (74 parts) dH20
63 µl (12.5 parts) buffer
63 µl (12.5 parts) 2 mM dNTPs
5 µl (1 part) 5U/µl Enzyme

Mix and store at 4°C for < 1 week.

2. For each run with a specific primer pair, make a primer-specific mix:

For separate 5 µM primers
1 part 5 µM primer 1
1 part 5 µM primer 2
7 parts master mix

For separate 5 µM primers
1 part 5 µM primer 1
1 part 5 µM primer 2
7 parts master mix

3. Add 1 µl of each sample DNA (for genomic DNA, 50 ug/ml or A260=1.0) to individual wells in a microtiter plate. Pipette 9 µl of the specific-primer mix into each well and mix by pipetting up and down. Load capillary tubes into the modular tops and aspirate 8 samples at a time by capillary action. Flame seal the loading end of the tubes, then seal other end. Place into the Air Thermo-Cycler and run at desired protocol. When reaction is complete, score each end of the glass tubes while still in the modular top, break glass and transfer directly into the gel wells.

Working Solutions

1. Primers and DNA are prepared in 1X TE':

50X TE' solution, pH 8.3 (500 mM Tris, 5mM EDTA)

10 m l 2 M Tris, pH 8.3
400 µl 0.5 M EDTA
dH2O to 40 ml

1X TE' solution, pH 8.3 (10 mM Tris, 0.1 mM EDTA)

200 µl 50X TE'	or	10 ml 50X TE'
dH2O to 10 ml		dH2O to 500 ml

2. Make 50 µM primer stocks with 1X TE'.

Make10X primers (5 µM) either separately or combined:

For 10X separate primers

40 µl (1 part) 50 µM Primer
360 µl (9 parts) 1X TE'

For 10X combined primers

40 µl (1 part) 50 µM Primer 1
40 µl (1 part) 50 µM Primer 2
320 µl (8 parts) 1X TE'

3. 10X Nucleotides (2 mM each dATP, dCTP, dGTP, dTTP)

250 µl 100 mM dATP (Sigma D4788)
250 µl 100 mM dCTP (Sigma D4913)
250 µl 100 mM dGTP (Sigma D5038)
250 µl 100 mM dTTP (Sigma T9656)
to 12.5 ml with dH2O

4. 10X Buffer

2.5 ml Tris, pH 8.3 (2 M stock)
0.5 ml BSA (50 mg/ml stock)
5.0 ml 40% Sucrose
1.0 ml 10 mM Cresol Red

Low Mg2+	200 µl (1M MgCl2) + 800 µl H2O
Medium Mg2+	300 µl (1M MgCl2) + 700 µl H2O
High Mg2+	400 µl (1M MgCl2) + 600 µl H2O

5. Enzyme diluent (10 mM Tris, pH 8.3, 2.5 mg/ml BSA)

50 µl 2 M Tris, pH 8.3
500 µl 50 mg/ml BSA
9.5 ml dH2O

Stock Solutions

All solutions are made from deionized, distilled water. No stir bars or pH meters are to be used in the preparation of stock or working solutions. Check pH by withdrawing 10 µl of solution and placing it on pH paper.

2 M Tris, pH 8.3

14.80 g Tris base (Sigma T1503)
12.28 g Tris HCl (Sigma T 3253)
to 100 ml with H2O

or

27.08 g TRISMA Preset, pH 8.3 (Sigma T5128)
to 100 ml with H2O

1 M MgCl2

20.3 g MgCl2 (Sigma M9272)
to 100 ml dH2O

or

Sigma M1028 (ready made)

50 mg/ml BSA

0.50 g BSA (Sigma A2153)
to 10 ml dH2O (use 15 ml tube)

10 mM Cresol Red

404 mg cresol red (Sigma C9877)
to 100 ml dH2O

40% (w/v) Sucrose

40.0 g sucrose (Sigma S5016)
to 100 ml dH2O

0.5 M EDTA, pH 8.3

18.6 g disodium EDTA (Sigma ED2SS)
10 ml 5 N NaOH (Baxter H369-1*NY)
to 100 ml dH2O

Rapid Cycle DNA Amplification – The 10 Most Common Mistakes

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Mistake #1.

Not having bovine serum albumin in the reaction. You will not get amplification in capillary tubes without BSA. Most buffers supplied by manufacturers of the enzyme do not include BSA. BSA is necessary to prevent surface adsorption/inactivation of the DNA polymerase on the large surface area of the capillary tubes. Yield increases with BSA concentrations up to 500 μg/ml in the reaction. Using gelatin gives a poor yield in capillary tubes. You can make up your own buffers. We recommend including 2.5 mg/ml BSA in the 10X buffer and 2.5 mg/ml in a 10X polymerase dilution. The grade of BSA is not critical. We use Sigma #A2153.

Mistake #2.

Using acetylated bovine serum albumin. It is expensive and does not work. Presumably, the same sites that are acetylated are those sites necessary to coat the glass walls and prevent polymerase inactivation.

Mistake #3.

Using Triton X-100. Some manufacturers of heat stable polymerases state that 0.1% Triton X-100 is needed for enzyme activation. Triton X-100 does activate some enzymes when BSA is not present and amplification occurs in microfuge tubes. However, Triton X-100 is not necessary when BSA is present. Furthermore, if Triton X-100 is added, yield substantially decreases in capillary amplifications that include BSA.

Mistake #4.

Adding polymerase to a microtiter plate before BSA. For convenience, many people mix reactions in a microtiter plate so they can be loaded simultaneously by capillary action into tubes already placed in modular tops. However, if the polymerase is added to a microtiter well before BSA, the polymerase can be adsorbed onto the plastic surface and not loaded into the capillaries. To prevent adsorption of polymerase during handling, we recommend diluting the polymerase to a 10X concentration with a diluent that includes BSA at 2.5 mg/ml. In addition, always block the well surface with BSA by adding the BSA-containing buffer before the polymerase. Microtiter plates that do not absorb protein can also be used and are available from Idaho Technology (microtiter plate, part

number 2590; lid, part number 2591) , but BSA is still necessary for the capillary tubes, whether glass or plastic.

Mistake #5.

Pulling tubes out near the denaturation temperature. If double stranded product is cooled rapidly (by pulling a tube out of an air cycler that is near denaturation temperatures), not all the product will reanneal and multiple apparent products may appear on gels.

Mistake #6.

Using excessive denaturation times. There is no reason for denaturation times longer than "0" sec at 94° C. The Tm of products in amplification buffer is around 85-90° C and complete denaturation of product at 94° C occurs faster than can be measured (< 1 sec. See Wittwer and Garling, 1991, BioTechniques, 10: 76-83, or Wittwer et al., 1994, in: The Polymerase Chain Reaction , Mullis, Ferre, and Gibbs, eds., pp. 174-181). The only possible exception is on the first cycle when high quality, complex genomic DNA is used as template. An initial denaturation of 5-15 sec at 94° C on the first cycle may allow more complete initial denaturation. However, extended times at high temperatures degrade DNA, and are particularly harmful in long product amplifications (CE Gustafson et al., 1993, Gene 123:241-244, and W.M. Barnes, 1994, PNAS, 91: 2216-2220).

Mistake #7.

Using nonstandard capillary tubes. The tubular metal-sheathed thermocouple that monitors temperature in the air cycler is precisely matched in thermal response to aqueous samples in the 10 μl capillary tubes sold by Idaho Technology. When nonstandard capillary tubes are used, the temperature of the sample will not correspond to the temperature indicated on the instrument readout. If you optimize a reaction in 10 μl tubes, and later run the reaction in larger tubes, you should not expect similar results. Larger tubes will not reach target temperatures without setting a hold time. If you insist on using larger or nonstandard tubes, you can monitor the sample temperature inside the tube with an IT-23 micro-thermocouple probe available from Sensortek (Clifton, NJ) and empirically adjust target temperatures and hold times. Be aware that some types of glass interfere with the reaction, presumably because ions on or near the surface of the glass are absorbed into the reaction buffer.

Mistake #8.

Forgetting to add a critical component. Accidental omission of polymerase, dNTP1s, or buffer components can be avoided by "master mixes" that include everything necessary for amplification except primers and template. Such a master mix, if sterile, lasts for 3-6 weeks at room temperature, >15 weeks at 4° C, and > 26 weeks at -20° C. Master mixes also minimize pipetting errors, particularly with small volumes.

Mistake #9.

Inappropriate Mg2+ concentration. Rapid cycling generally requires higher magnesium concentrations than slow cycling. For example, whereas 1.5 mM magnesium chloride is standard in slow cycling, 2-4 mM is more typical for rapid cycling. With 2-4 mM magnesium chloride, excellent yield and specificity can be obtained with annealing times of "0" sec. Magnesium chloride is hygroscopic and it may be difficult to prepare accurate solutions from the solid salt. We use a 1 M solution of magnesium chloride available from Sigma (#M1028).

Mistake #10.

Poor temperature/time optimization. Rapid cycle temperature/time parameters are very different from slower cyclers. It is a mistake to directly transfer a protocol like, "1 min at 94° C, 2 min at 55° C, and 3 min at 72° C," to a rapid cycler. Denaturation should be set at 94° C for 0" sec. The annealing time should almost always also be set at "0" sec. The extension temperature should be 70-74° C. The extension time should be "0" sec for products up to 100 bp, 5-15 sec for products up to 500 bp, and about 30 sec for a 1000 bp product. Most amplifications with 20-mer primers will work well using 3 mM MgCl2 at an annealing temperature of 50° C. Rapid cycling makes it feasible to rapidly test all combinations of 3 different annealing temperatures (40° C, 50° C, and 60° C) and 3 different Mg concentrations (2 mM, 3 mM, and 4 mM).

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Capillary Tube Handling with the Rapidcycler

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One of the biggest concerns for new users of air-cyclers is the handling and sealing of glass capillary tubes. While they are a bit more difficult to use than the traditional microcentrifuge tube, the rapid cycle times and temperature homogeneity made possible by the capillaries makes them more than worth the extra trouble. After a little practice, you may wonder why you ever worried.

Single Tube Handling

Mixing the Sample

You can mix your reaction in any sort of container, I use low protein absorbing microtiter dishes (IT#2590). Take care at the mixing step as one of the most common causes of reaction failure is forgetting a component of the reaction (see "The 10 most common mistakes", Rapid Cyclist 2:11-12). The chances of leaving something out can be reduced by making up "master mixes" that contain everything but primer and template. The mix can be stored at 4° C for up to 3 months (see "Reaction mixes and buffer recipes", Rapid Cyclist 2:9).

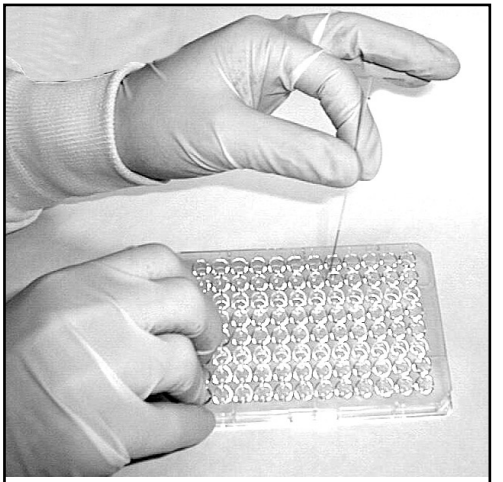

Figure 1. Tipping the capillary tube sideways to increase the rate of liquid uptake.

Loading the capillary

Glass capillary tubes are easily loaded by capillary action. You can increase the rate of liquid uptake by tipping the capillary tube sideways (figure 1). You can also load the capillaries using a Drummond microaspirator (IT#1690) to draw the reaction mix up into the tube, or you can use a pipetman to directly inject sample into the tube (Figure 2). The 10 ul size tubes hold 2.2 ul/cm and can be used for reaction volumes from 5 to 15 ul. The 10 ul capillaries come to temperature so quickly that they require no holds at denaturation or annealing. The 50 ul tubes hold 9 ul/cm and are useful for reaction volumes from 15 to 70 ul. These tubes require a 15 second hold at the denaturation and annealing temperature.

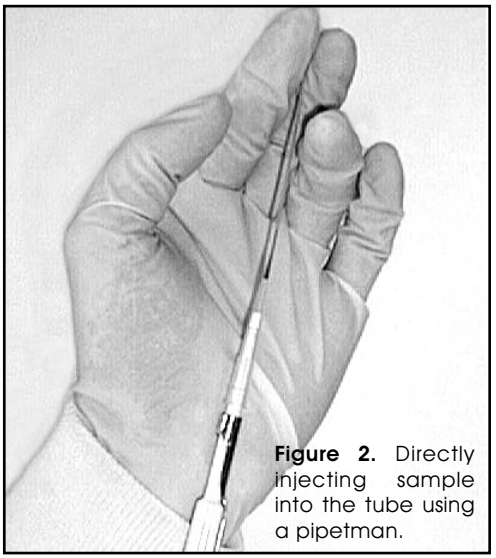

Figure 2. Directly injecting sample into the tube using a pipetman.

Sealing the capillary

The glass capillaries sold by Idaho Technology are made out of a high sodium, low melting temperature glass. This makes them very easy to flame seal with just about any flame, They can be sealed with a Bic lighter (Figure 3), a Bunsen burner, a candle, or, my favorite, a Blazer mini propane torch (IT#2721).

After the capillary is loaded, tip the tube to center the liquid. Hold the tube in the center and place the end just into the flame. Rotate the tube in the flame by rolling it between your thumb and index finger. You should be able to see the glass slowly close in on itself. Try to avoid leaving the tube in the flame too long, as you can end up with a big ugly glob of glass which will not fit into the holder . This is more

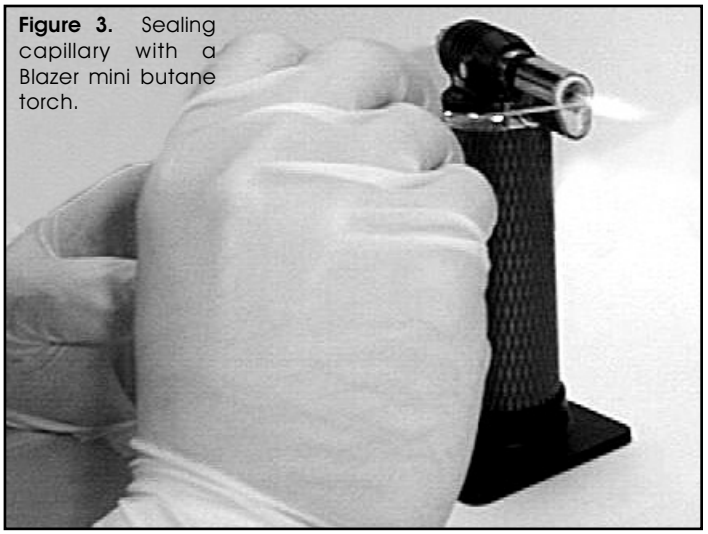

Figure 3. Sealing capillary with a Blazer mini butane torch.

likely in very hot flames. Cutting down the air to the flame will cool these burners down and make the capillaries easier to seal.

You can confirm that the end is sealed by looking carefully at the end for a continuous wall of glass around the end. You can also confirm sealing by blowing on the hot end of the capillary and watching to see if the liquid moves toward the end of the capillary as the glass cools (This is more dramatic for the first seal than the second).

Repeat the sealing process on the other end and then insert the tube into the capillary holding module. A module rack (IT#1735) makes these manipulations easier.

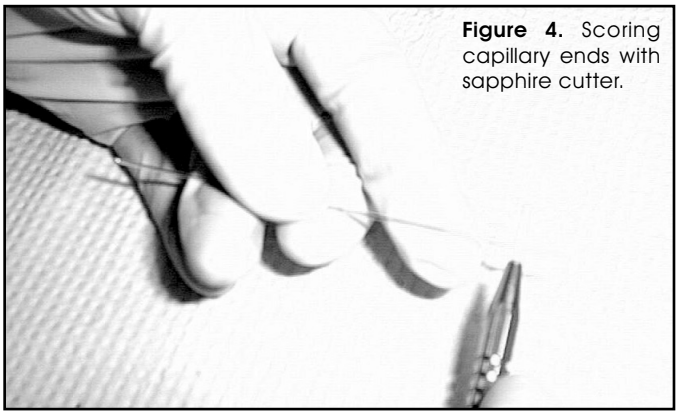

Figure 4. Scoring capillary ends with sapphire cutter.

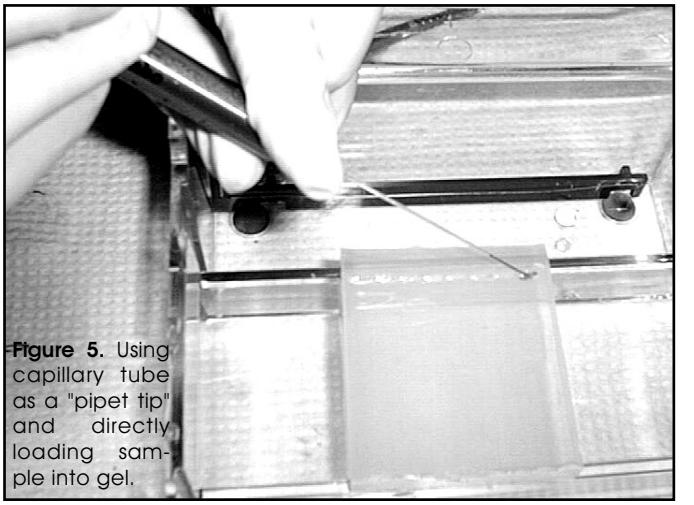

Figure 5. Using capillary tube as a "pipet tip" and directly loading sample into gel.

Sample Recovery

After your reaction is done you pull the tube from the module, lightly score the two ends with a sapphire knife (Figure 4, IT#1691) and break off the ends. The capillary tube then becomes a 3pipet tip2 for the Drummond microaspirator (IT#1690) and can be used to directly load your sample into a gel (Figure 5), or into a storage tube.

Beware, the pressure caused by sliding the capillary into the microaspirator can cause your sample to be blown out of the tube. This is easily prevented by dialing the microaspirator back a bit as you insert the capillary tube. The silicon tips of the microaspirator wear out quite quickly, so if your microaspirator stops working try replacing the tip (IT#1870).

Multiple Tube Handling

Once you get single sample handling down, you may want to try some of these "advanced" multiple sample handling tricks.

Eight Sample Handling

When sample modules are made with microtiter spacing it is possible to mix up eight samples at a time in a microtiter dish and draw them up simultaneously by capillary action (Figure 6). All eight samples can be centered by tilting the module and then the tubes can be sealed by passing the tubes through a flame one at a time (Figure 7). Once the reaction is done you can score all eight tubes at once by lightly drawing the sapphire knife across the top of the module (Figure 8) and then breaking off each tube top (Figure 9). Press the module down to the other end of the capillary tubes and repeat the scoring and breaking.

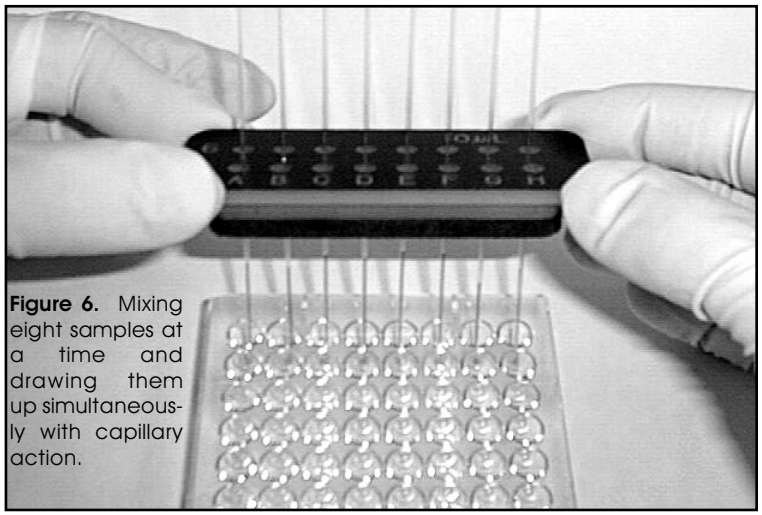

Figure 6. Mixing eight samples at a time and drawing them up simultaneously with capillary action.

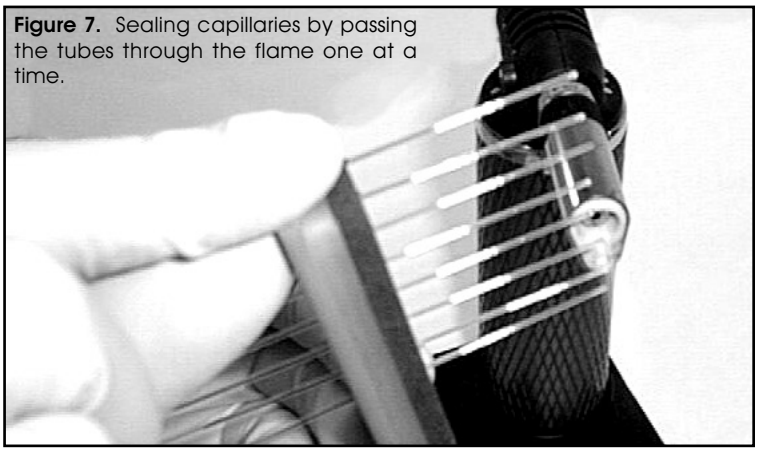

Figure 7. Sealing capillaries by passing the tubes through the flame one at a time.

Sixteen Sample Handling

After mastering the eight sample tricks, you may want to try 16 at a time. All sixteen tubes in the module can be filled simultaneously by capillary action. After centering the samples the two rows of eight tubes can then be staggered off from each other by pressing the tubes down on a bench top. The bottom of the first row of eight tubes, and the top row of the second row of eight can then be sealed one at a time by passing through the flame. The staggered rows can then be switched and the remaining two ends can be sealed. After the reaction is done the ends can be scored as in the eight sample example.

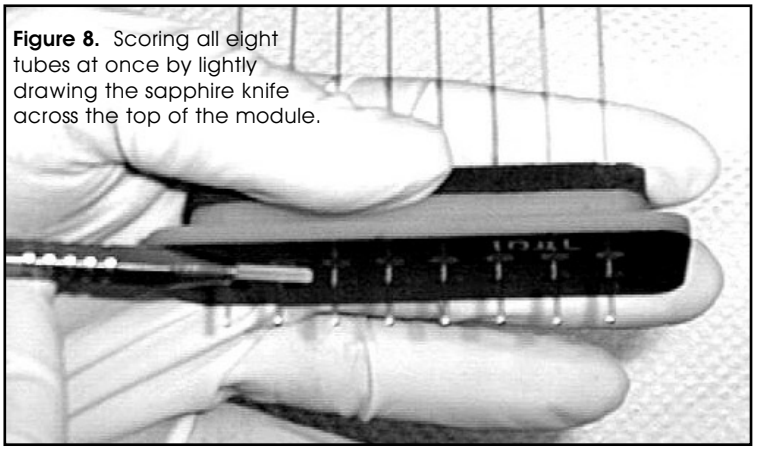

Figure 8. Scoring all eight tubes at once by lightly drawing the sapphire knife across the top of the module.

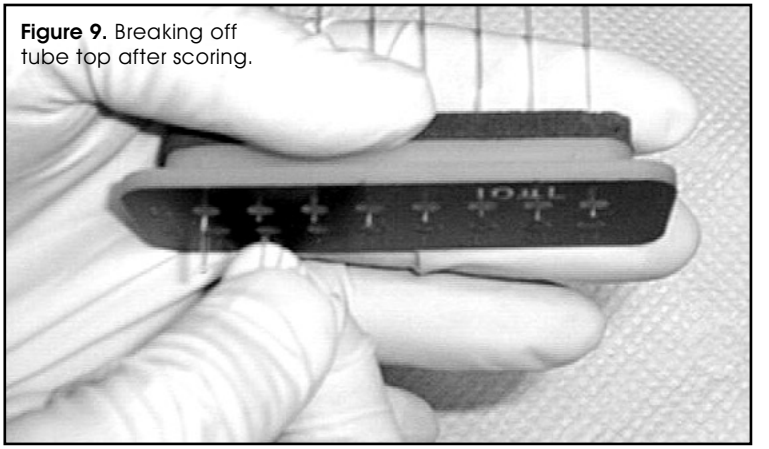

Figure 9. Breaking off tube top after scoring.

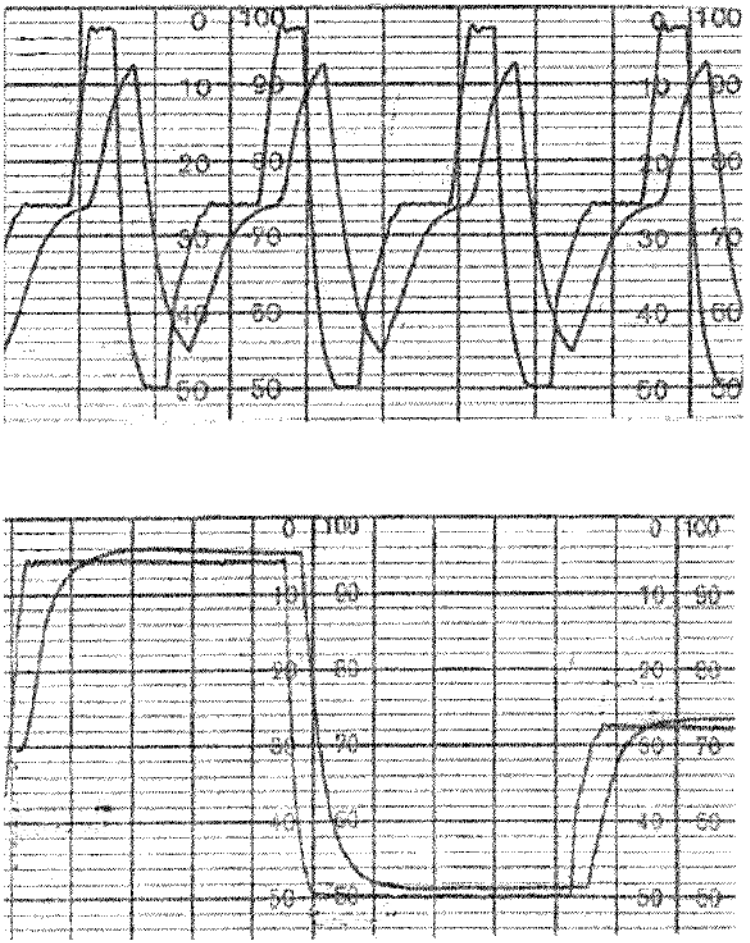

Figure 2. Temperature traces of the hold method (2B) versus the over heat and under heat method (2A). Traces are of air temperature and actual sample temperature. Notice how the sample temperature always lags behind the air temperature, and how the over/under heat method brings the sample to temperature more quickly.

Use of Thin Walled Microcentrifuge Tubes with the RapidCycler

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The development of thin walled micro test tubes makes it possible to combine the speed of the air cycling with the convenience of that "universal vessel" of molecular biology, the microcentrifuge tube. While the Rapidcycler was developed for use with glass capillaries, it provides excellent results with thin walled microcentrifuge tubes. Using modified sample modules, the Rapidcycler can hold up to 48 micro test tubes Figure 1 shows that all 48 positions give a clean, bright, 500 bp product in a DNA amplification from Human genomic DNA.

Thin walled micro test tubes have many advantages over capillary tubes. First, handling of the sample tube is much simpler; reactions can be made up in the micro test tube, no heat sealing is required, concern about breaking the tubes is eliminated. Second, there is no need to adjust buffers or protocols. The buffers that manufacturers provide with their thermostable polymerases work in these tubes without modification. Published protocols developed in heat block instruments seem to transfer more readily to the Rapidcycler when micro test tubes are used.

The thermal properties of thin walled microcentrifuge tubes are much better than their thick walled ancestors, but they are still no match for a capillary tube. Using thin walled microcentrifuge tubes requires a sacrifice in speed and in sample temperature uniformity. A 10 μl reaction that would take 15 minutes in a capillary tube, takes 35 minutes in a thin walled microcentrifuge tube, a 50 μl reaction that would take 20 minutes in a capillary, takes 50 minutes in a microcentrifuge tube.

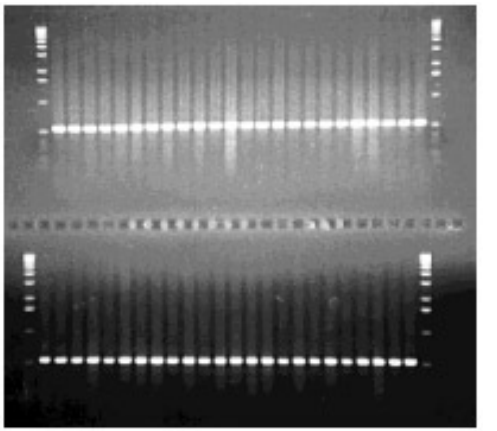

Figure 1. Amplification of a 500 bp target from human genomic DNA in all 48 sample positions of the Air Thermo-Cycler. Reactions volume was 50 μl, no oil overlay. Reactions contained Idaho Technology medium buffer, 200 μM each dNTP, 5 μM each primer (RS/KM), 50 ng human genomic DNA. Cycling parameters were 96° for 30 seconds, then 30 cycles of 96° for 30 seconds, 55° for 30 seconds, 75° for 20 seconds.

Because the Rapidcycler was developed for capillary tubes the temperature values that you program into the machine, and the temperatures displayed during cycling, reflect what the temperature would be in a 10 μl capillary. When using microcentrifuge tubes you must modify the program parameters to compensate for the thermal differences between capillaries and microcentrifuge tubes.

Thin Walled Microcentrifuge Tube Cycling protocols for the Rapidcycler

There are two possible approaches when using microcentrifuge tubes. You can set the machine to the temperature you want, and wait for the microcentrifuge tube to get to that temperature (Figure 2B) This is what the slower heat block cyclers do). This method is slow, but it assures you that no part of your sample is ever over the target temperature. A faster approach is to overheat and under heat the air. This brings the sample to temperature more quickly (Figure 2A). The faster heat block instruments do this), but some parts of your sample may be slightly above or below the target temperatures.

I have had good success with the faster overheat and under heat approach. The following protocols have been successful with a variety of primers and DNA sources. If you prefer the sit and wait approach 10 μl samples require 40 second holds at denaturation and annealing, 50 μl samples 60 second holds at denaturation and annealing. Elongation requires 25 nucleotides per second plus about 15 seconds.

10 μl Reactions

Predenature: 98°C for 10 seconds

Cycle:	Denature	98°C for 10 seconds
	Anneal	40°C to 60°C for 10 seconds (as appropriate for your primers)
	Extend	74°C for 25 nucleotides per seconds

50 μl Reactions

Predenature: 96°C for 30 seconds

Cycle:	Denature	96°C for 30 seconds
	Anneal	40°C to 60°C for 30 seconds (as appropriate for your primers)
	Extend	74°C for 25 nucleotides per seconds

Optimization of Reactions in Thin Walled Microcentrifuge Tubes

The same optimization protocol that has been recommended in capillaries (Optimizing Rapid Cycle DNA Amplification Reactions, Rapid Cyclist 1:1-5,1992) has provided excellent results in thin walled microcentrifuge tubes.

Optimal reaction conditions are found by running amplifications at 40°C, 50°C and 60°C with 2 mM, 3 mM and 4 mM MgCl2 at each temperature. This allows you to test 9 different stringencies, while only requiring you make up three different reaction mixes.

I have used this optimization protocol successfully with Idaho Technology buffers (low, medium and high MgCl buffers), Promega 10X Taq buffer and Stratagene 10X Pfu buffer.

Are Mineral Oil Overlays Required?

The thin walled microcentrifuge tube holders for the Rapidcycler put the entire tube inside the reaction chamber. This keeps the whole tube at the same temperature and thus reduces condensation. A small amount of condensation occurs on the leeward side of the tubes, but I have not found this to be a practical problem, even for 10 μl reactions. While a little mineral oil does stop this condensation, in general, oil is not needed for 10 μl reactions.

50 μl reactions show minimal condensation, but will occasionally pop open during reactions if no oil is used. The frequency with which this occurs seems to vary with reaction buffer and with tube manufacturer, so you may wish to experiment with your particular combination.

Real Versus Set Temperatures

The actual sample annealing temperature may not be important to you if you optimize the reaction experimentally as recommended above. If you do need a particular annealing temperature, the value you should set can be calculated using the equations in figure 4. I have provided graphs for 10 μl reactions with a 10 second hold (Figure 4A) and for 50 μl reactions with a 30 second hold (Figure 4B).

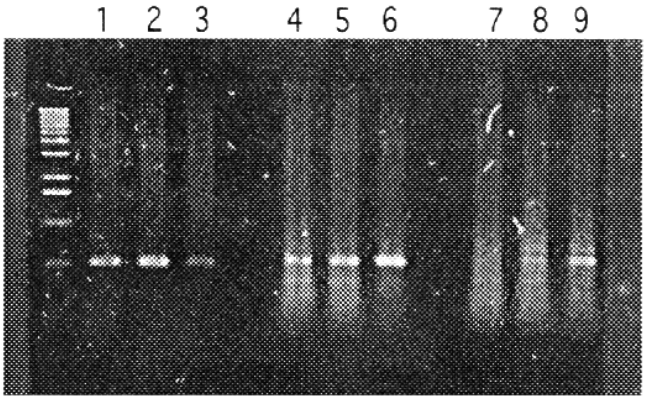

Figure 3. Optimization of RS/KM primer pair in microfuge tubes. Lanes 1-3: 60°C annealing, 4, 3 and 2 mM MgCl. Lanes 4-6: 50°C annealing, 4, 3 and 2 mM MgCl. Lanes 7-9: 40°C annealing, 4,3 and 2 mM MgCl. 10 μl reaction volume, no oil, 10 sec. holds at annealing and denaturation.

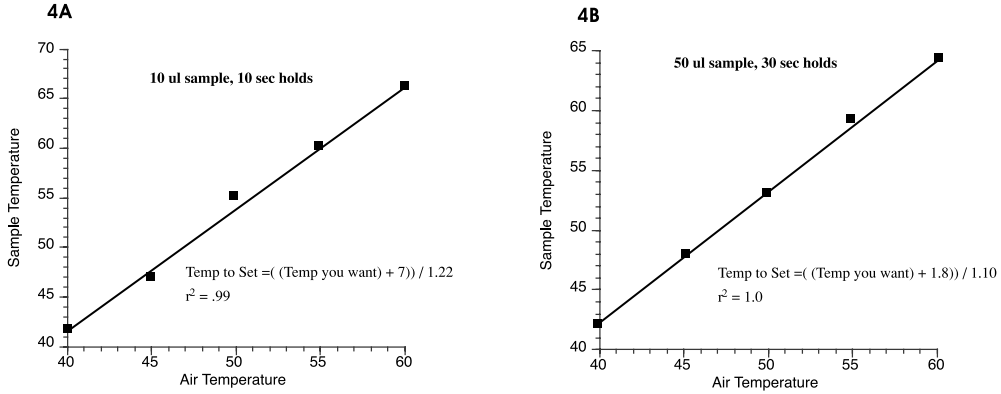
4A
10 ul sample, 10 sec holds
Sample Temperature
Air Temperature
Temp to Set =((Temp you want) + 7)) / 1.22
r2 = .99
4B
50 ul sample, 30 sec holds
Sample Temperature
Air Temperature
Temp to Set =((Temp you want) + 1.8)) / 1.10
r2 = 1.0

Figure 4. Linear relationship between the temperature programmed into the air cycler and the actual sample temperature for thin walled capillary tubes. 4A: 10 μl samples, 10 second holds, no oil overlay. 4B: 50 μl samples, 30 second holds, no oil overlay.

Direct Sequencing of Long PCR Products

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Introduction

Vitamin B12 is an essential cofactor of many non-photosynthetic eukaryotes. It is synthesized by prokaryotes and archebacteria both aerobically and anaerobically. In Salmonella typhimurium the anaerobic pathway is dependent on at least 30 genes. Several of these genes also occur in Escherichia coli, allowing synthesis from intermediates.

In spite of the fact that over 1% of the Salmonella genome is dedicated to B12 synthesis, cells unable to make the cofactor do well anaerobically under laboratory conditions. Only a few B12 dependent pathways are known and none seem essential. For example, the eut enzymes enable growth on ethanolamine as a source of carbon or nitrogen; the pdu regulon allows utilization of propanediol as a carbon source; and the MetH protein provides an alternate route for the final step in methionine biosynthesis.

Knowing the sequence of the eut operon forms a fundamental part of our strategy in characterizing the synthesis and importance of B12 in Salmonella. I have not been able to clone eut using standard techniques, suggesting that minor variations in its expression levels may have dramatic effects on the well-being of the cell. Instead, I have chosen to amplify portions of the operon from the genome and to sequence these PCR products directly.

Sequencing Strategy

Direct PCR sequencing has the advantage of blending Taq polymerase-induced errors into the background. However, linearized double-stranded template yields short, dirty "reads" with many premature stops. The technique is usually avoided in favor of sequencing cloned amplified DNA. Such inserts often contain polymerization mutations. Two and sometimes three independent clones must be sequenced to determine the primary structure with confidence. This, together with the overhead of plasmid preparation, increases the time required.

Recently, a fast and efficient method for DNA strand separation based on magnetic bead technology became commercially available (Dynal; 5 Delaware Drive, Lake Success, NY 11042; 800 638-9416). This allows exceptionally clean direct PCR sequencing using single-stranded templates. In addition, by optimizing for long amplification products, less time is spent preparing DNA.

Mispriming & Parasites

I routinely amplify product in the range 3-5 Kb. A major problem when generating molecules of this size is the tendency of non-specific smaller products to deplete reactants. Such parasites arise through false priming events and become favored, as efficiency per cycle is inversely correlated with product size. The short dwell times used by the Air Thermo-Cycler during annealing discourage false priming. Nevertheless, single primer controls should always be run to verify that a given product is dependent on both primers.

I eliminate primer dimer formation and false priming prior to the first denaturation step by including TaqStart antibody (Clonetech, Catalog #5400-x; 4030 Fabian Way, Palo Alto, CA 94303-4607; 800 662-2566). This temporarily inactivates the polymerase, which reactivates as increasing temperature denatures the antibody. To use the reagent, combine 1 volume Taq polymerase (5 units/ml), 1 volume of TaqStart (7 uM) and 10.5 volumes of enzyme dilution buffer (2.5 mg/ml bovine serum albumin [BSA] in 10 mM Tris pH 8.3). This is then used as normal Taq polymerase stock at 0.4 units/ul.

Primer Design

Best results always correlate with well designed primers (20-30 nucleotides with approximately 50% G+C content; no 3' terminal complementarities; no internal palindromes; no runs of G or C near 3' end). Primers should be "balanced " in the sense that overall lengths and compositions are about the same. I often include 5' tails for special purposes and find little, if any detrimental effect if the 3' end is at least 20 bases long. Trailing sequences can be amazingly long.

Reamplification

I usually use reamplified template DNA for sequencing. I first prepare a starter by amplification of genomic DNA (either purified, crude or encapsulated in cells). Subsequent template preparations are reamplified from a 1:100 dilution of the starter. This is especially important when genomic DNA (prepared according to Ausubel, F.A. et al. (eds), 1990, Current Protocols in Molecular Biology, Greene Publishing and Wiley-Interscience, New York, pp. 2.4.1 - 2.4.2) is used in the primary reaction.

I have also had excellent success using cells scraped form a plate in place of genomic DNA. In this case, I either simply touch the cells directly to the reaction mix, or resuspend them in 50 uL TE (10 mM Tris, pH 8.3; 1 mM EDTA), heat 2' at 95°, spin down and use the supernatant, diluted zero to 100-fold. The remainder can be frozen for future use.

More Tricks for Large Products

Purified genomic DNA should never be preboiled. Too many nicks are introduced and long products are more difficult to amplify. Preceding a primary genomic amplification with a 30 sec hold at 94° and following PCR with a 5 min hold at 72° improves yield. When characterizing a new primer pair, I always optimize according to the simple "3 x 3" scheme of Rasmussen (Rapid Cyclist vol .1, no. 1, 1-5, 1992). This takes only a couple of hours and pays great dividends.

PCR Reaction for a Typical Analytical Amplification

Use 10 - 15 uL of the following in a single capillary. For a preparative run, scale by 5 and load into 6 capillaries.

A Typical Amplification Program

For products longer than 1 kb, assume an elongation rate of 20 bases/sec for the elongation time. The annealing temperature can vary from 40° to 65°, and is determined empirically in a "3 x 3" optimization. For products longer than 3 kb, a denaturation time of 5 sec frequently improves the yield. If primers are poorly balanced or imperfectly match their sites, a ramp constant "S" of 6 will sometimes help.

For single product preparative runs, use Wizard PCR Prep (Promega; 2800 Woods Hollow Road, Madison, WI 53711-5399; 800 356-9526) for rapid cleanup. Elute with H2O and store at -20°. When more than one band is present, excise the correct one and purify with GeneClean (Bio 101; PO Box 2284, La Jolla, CA 92038-2284; 800 424-6101). Again, elute with H2O and store frozen.

8 uL water
2 uL 10X PCR Reaction Buffer
2 uL 4 dNTP's, each at 2 mM
2 uL primer 1 at 5 uM
2 uL primer 2 at 5 uM
2 uL DNA, diluted 1:100
2 ul Taq + TaqStart (see above)

Total = 20 uL

10X PCR Reaction Buffer: 500 mM Tris, pH 8.3, 2.5 mg/ml BSA, 5% Ficoll and 10 mM Cresol Red. MgCl2 added to 10, 20 or 30 mM.

1. Hold 30 sec at 94°

2. Cycle parameters
(as they occur on Air Thermo-Cycler screen):

D94	A50	E72	C30	S9
d0	a0	e1'0"		

3. Hold 5 min at 72°

Magnetic Strand Separation & Purification of Both DNA Strands

There are several methods for preparing single-stranded DNA from PCR products, such as asymmetric amplification, exonuclease digestion and magnetic separation. I prefer the last as it is fast and the magnetic beads lend themselves to a number of other techniques. It requires that one and only one of the two PCR primers be biotinylated. Generally, there is little additional cost for this service.

DynaBeads or "Beads" - DynaBeads M-280 Dynal

"Acetate Solution" - Potassium acetate, pH 4.8
(5 M acetate, 3 M K)
294 g KCH3CO2
115 ml HCH3CO2
H2O to 1 liter (no need to check pH)

HBWB - high-salt "Binding & Wash Buffer"
10 mM Tris, pH 7.5
1 mM Na2 EDTA
3 M NaCl

TE
10 mM Tris, pH 7.4
1 mM Na2EDTA

Glycogen Solution - 20 mg/ml
Boehringer Mannheim, Catalog # 901-393

The protocol yields two DNA strands, separated and purified in a manner suitable for direct sequencing. The "W" strand is biotinylated and the "C" is its complement. Preparation of the "W" strand is a modification of the Dynal protocol which conserves beads with no apparent sacrifice in yield. The method for preparing "C" strand is new . Typically, 5 uL of either strand preparation is used in a single sequencing reaction.

1. Wash the beads:
vortex bead stock
add 10 uL beads to 20 uL HBWB in tube "W" vortex
magnetically separate, discard supernatant
resuspend beads in 30 uL HBWB

2. Bind DNA
add 5 -20 uL PCR product to bead pellet mix by flicking
incubate 15 min RT with occasional resuspension
magnetically separate, discard supernatant
resuspend beads in 40 uL HBWB
magnetically separate, discard supernatant

3. Denature DNA:
load tube "C" with 30 uL 3 M acetate solution
resuspend beads in 15 uL 0.2 N NaOH
incubate 15 min x RT with occasional resuspension
magnetically separate, add supernatant to tube "C"

4. More strand separation - repeat 3x
resuspend beads in 50 uL 0.2 N NaOH
magnetically separate, add supernatant to tube "C"
resuspend beads in 40 uL HBWB
magnetically separate, discard supernatant
resuspend beads in 50 uL TE
magnetically separate, discard supernatant

5. "W" strand cleanup
resuspend beads in 25 uL H2O
store at -20 °

6. "C" strand cleanup
add 1 uL glycogen to tube "C" and mix well
add 500 uL 95% ethanol - mix well
30 min x ice
microfuge 10 min
wash once with 400 uL 70% ethanol
draw off ethanol in vacuum jar; avoid fully drying pellet
resuspend in 25 uL H2O
store at -20°

Sequencing Reactions

This is a synopsis of my current sequencing methods. I use "Sequenase Version 2 with Pyrophosphatase" (USB, Catalog # 70175) and the "Manganese Reagent" Sequenase Kit (USB, Catalog # 70130), which employs extensions and slight modification of commonly used dideoxy technology.

This protocol will yield sufficient material for fully redundant loading of 1.5 uL samples on two gels. "Mix" quantities are given for a single primer/template pair. Multiply amounts by the number of such sequences plus one. Wherever temperature blocks are called for, each cavity used is filled with water.

1. Annealing - combine in a small Eppendorf vial:
7 uL DNA at 0.1 - 1 ug/mL
1 uL primer at 5 uM
1 uL 10X MOPS: included in kit
1 uL 10X Mn Solution: included in kit
Total = 10 uL
2 min at 65° - temp. block
30 min at 42° - small oven or temp. block

2. Extension - add:
5.5 uL EMix
Total = 15.5 uL

0 -5 min at room temperature (Shorter times allow reads closer to template. 5 min is the norm.)

During this time, add 2.5 uL termination mixes to a preheated (37 ° C) mini tray. Distribute each mix to its own column, filling as many wells as there are reactions. Each row corresponds to one primer/template pair and can be color coded on the reverse side of the tray.

"Manganese Reagent" Kit
USB, "Mn2+ Reagent Kit for DNA Sequencing", catalog # 0130

"Enzymes"
USB, "Sequenase Version 2 with Pyrophosphatase", catalog # 70175

EMix
1.6 uL H2O
1.0 uL 0.1 M DTT: included in kit
0.4 uL Sequence Labeling Mix (incl. in kit), diluted 1:5 with H2O
0.5 uL labeled dATP (32P, 33P, or 35S)
2.0 uL enzymes
Total = 5.5 uL

"Mini Trays"
InterMountain Scientific, 1610 S. Main,Suite H, Bountiful, UT 84010, (801) 298-7884;
"Micro Well Mini Tray", cat. #438733.

TdT Mix
3.4 uL H2O
0.3 uL 4 dNTP's, each at 2 mM
(Pharmacia, "Ultrapure dNTP Set", cat. # 272035)
0.3 uL terminal deoxynucleotidyl transferase @20 u/uL (BRL, cat. # 8008SB)

Total = 4.0 uL

3. Termination - add to each well of appropriate row:
3.5 uL extension reaction
Total in each well = 6 uL
10 min at 42° - small oven or temp. block

4. TdT Extension - optional; used to resolve premature stops.
Add to each well:
1 uL TdT Mix
Total in each well = 7 uL

5. Electrophoresis: place tray in vacuum jar, evacuate 15 min.
add to each well:
4 uL stop solution: included in kit
Total = ~6 uL
3 min at 75° - place covered tray under slug of 75° temperature block load 1.5 ul sample per well

Rapid PCR Fingerprinting of Bacterial Genomes with REP Primers in Capillary Tubes Using the Air Thermo-Cycler.

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Introduction

DNA fingerprinting of genomes using PCR methods has been intensively used during the last years to characterize genomic diversity and to search for specific DNA markers (1-9). Different DNA banding patterns have been obtained using primers with length ranging from 8 to 25 nucleotides containing either arbitrary (2, 3, 5, 8, 9) or specific sequences (4, 6). In particular, the use of bacterial Repetitive Extragenic Palindromic sequences (REP) and Enterobacterial Repetitive Intergeneric Consensus (ERIC) have been proved to be practical and appropriate to fingerprint a number of different bacterial species (4, 6). Although REP and ERIC primers do not lead to amplification patterns as complex as those obtained with the random primed DAF (1), visualization of amplified DNA fragments can be easily achieved by agarose gel electrophoresis/ethidium bromide staining. Thus, classic REP and ERIC PCR amplifications may be efficiently used to characterize Gram-negative and Gram-positive bacterial genomes in a 10 hours experimental procedure. Here, we report a simple, rapid and reproducible protocol to perform REP DNA amplifications in 2 h using capillary tubes in air thermo-cyclers.

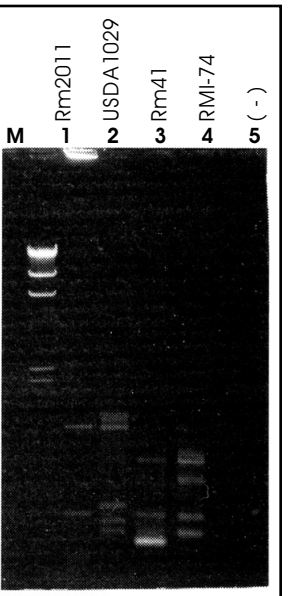

Figure 1. amplification patterns of four distinct R. meliloti strains using whole bacteria as the source of DNA in the Air Thermo-Cycler (ATC). Lane 1, Rm 2011; lane 2, Rm 1029; lane 3, Rm 41; lane 4, Rm 1-74 and lane 5, control without template. Molecular weight marker: lambda/HindIII.

Methods

The amplification mixture composition was as follows: 50 mM Tris, pH 8.3; 500 μg/ml BSA; 3mM MgCl2 (1x high magnesium buffer-Idaho Tech.); 200 μM dNTPs; 1U Taq DNA polymerase (Promega Corp.); 15 μM of each REP primers; and 1 μl of bacterial cells from a fresh isolated colony as the source of DNA template in a final volume of 25 μl. The cycling conditions were as follows: 95°C for 5 min; 30 cycles at 94°C for 10 sec., 40°C for 10 sec. and 65°C for 2 min.; 1 final step at 65°C for 4 min. All PCR amplifications were carried out using an Idaho 1605 Air Thermo-Cycler (ATC) in 25 μl capillary tubes. Ten μl of each sample were electrophoresed in 0.8-1.5% agarose gels added with 150 μg/l ethidium bromide.

Results

We set up the experimental conditions using 4 strains of the soil bacteria Rhizobium meliloti, Rm 2011 (Dr. J. Dénarie, Toulouse, France), Rm USDA 1029, Rm 41 (Dr. A. Kondorosi, Paris, France) and Rm 1-74 (Dr. A. Pühler, Bielefeld, Germany). The rapid transfer of heat in the capillary sample container allowed the shortness of amplification cycles from the required 600 sec. in metal block thermo-cyclers (MBTC), to 140 sec. in the ATC. Thus, the whole protocol could be carried out in less than 2h using either 1 μl of intact bacterial cells or purified DNA as template. The obtained DNA amplification patterns were all different among the strains (Fig 1) and allowed us to identify any of them in subsequent screenings. To validate this protocol designed for the ATC, we compared REP amplifications in MBTC with those obtained with the conditions here described. Figure 2 shows that DNA amplification products were comparable and tended to parallel each other when BSA was present in the reaction indi-

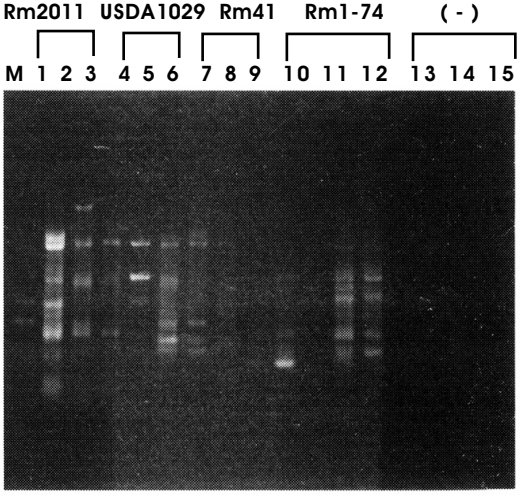

Figure 2. REP amplification patterns using metal block (MBTC) and capilary air thermo cyclers (ATC). Lane 1, Rm 2011 MBTC: lane 2 Rm 2011 MBTC/BSA; lane 3 RM 201 ATC/BSA; lane 4, Rm 1029 MBTC; lane 5 Rm 1029 MBTC/BSA; lane 6 Rm 1029 ATC/BSA; lane 7, Rm 41 MBTC; lane 8 Rm 41 MBTC/BSA; lane 9 Rm 41 ATC/BSA; lane 10, Rm 1-74 MBTC; lane 11 Rm 1-74 MBTC/BSA; lane 12 Rm 1-74 ATC/BSA; lanes 13, 14 and 15, controls without template for MBTC, MBTC/BSA and ATC/BSA, respectively. Molecular weight marker: pUC 9/HaeIII.

cating that BSA has additional effects other than the enzyme protection in the capillary system. Moreover, the presence of BSA allowed the amplification of DNA from the strain Rm 1-74. Total DNA preparation from these rhizobia was systematically contaminated by a yet unknown pigment which strongly inhibited conventional PCR amplifications.

The system here described for the characterization of bacterial genomes is fast, reproducible, strain specific, and suitable for amplification of samples containing natural PCR inhibitors not removed during the cell heating or template DNA preparations. The high number of individual isolates in strain collections represent a limiting factor during the selection of molecular characterization methods. The possibility to obtain reproducible DNA fingerprints in a short time represents a valuable alternative for programs of germoplasm characterization.

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Comparison of Different PCR Cycler Machines for Rapid and Sensitive Detection of Pathogens

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Abstract

In order to find a rapid PCR method to detect bacterial pathogens, we compared different PCR cycler machines. The total cycle time to complete the PCR amplifications were: 5 hours in the BioOven (BioTherm Co.) PCR cycler; 1.5 hours in the MiniCycler (MJ Research, Inc.), 2.5 hours in the Perkin Elmer Cycler 480; 3 hours in the PHC-2 Cycler (Techne Inc.), but only 30 minutes in the 1605 Air Thermo-Cycler (Idaho Technology). Using the 1605 Air Thermo-Cycler with our rapid and simple sample preparation method, the total detection and identification time was 1.5 - 2 hours including 30 minutes for the PCR cycles and 40 minutes for electrophoresis. Eight bacterial species have been tested with this protocol in the 1605 Air Thermo-Cycler, all of them gave good results.

Introduction

Rapid and specific methods for detection and identification of pathogens are essential for food safety and clinical diagnosis of human and animal diseases. Antibody-based test methods are the most often used technique. However, PCR based methods should be faster and more specific. A traditional PCR protocol takes about 5 hours in Perkin Elmer Cycler 480. Previously, we reported a protocol for the PHC-2 cycler machine (Techne Inc., Princeton, NJ), which shortened detection time to 3 hours (2,3,4,5). In this article, we report the results of comparison of different PCR cycler machines for the rapid and sensitive detection of pathogens.

Materials and Methods

The bacterial cells were collected from liquid cultures by centrifugation. The cells were washed twice with phosphate buffered saline (PBS), distilled water (dH2O), and resuspended in dH2O at 107 cells per μl. Just before the PCR assay, the samples were diluted to the desired cell concentration of 105 CFU in 50 to 100 μl of 1% Triton X-100. The cells were then heated at 100°C for 5 minutes, immediately cooled in ice water, and tested by PCR without isolation of the DNA. Two μl of above sample were added to 23 μl of a PCR mixture.

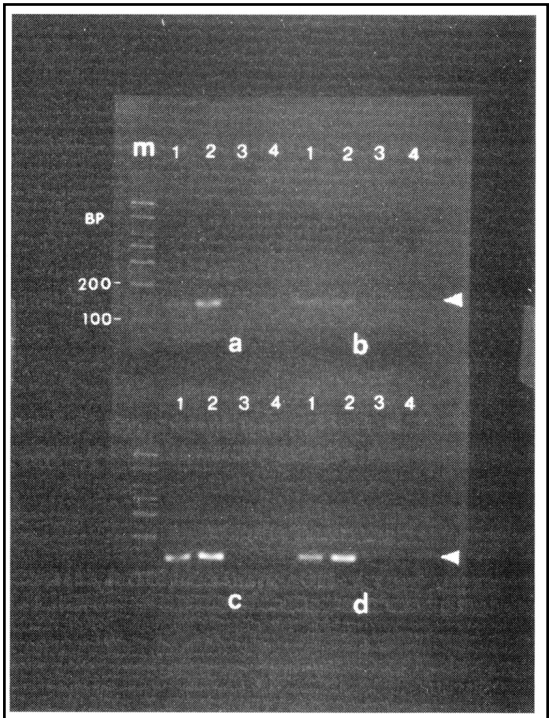

Figure 1. PCR results in different thermal cycler machines. PCR primers are specific for Mycoplasma gallisepticum (unpublished data). The PCR product is 138 base pair DNA fragment. 3% agarose gel was used for the electrophoresis. Lane m: molecular size marker. Lane 1: Mycoplasma gallisepticum strain K23. Lane 2: M. gallisepticum strain K730. Lane 3: Mycoplasma synoviae strain FMT. Lane 4: H2O for control. Panel a: The MiniCycler was used with a total cycle time of 1.5 hours. Panel b: The BioOven was used with a total cycle time of 5 hours. Panel c: The PHC-2 Cycler was used with a total cycle time of 2.8 hours. Panel d: The 1605 Air Thermo-Cycler was used with a total cycle time of 30 minutes.

For the BioOven, MiniCycler, Perkin Elmer Cycle 480, and PHC-2, the PCR mixture contained 50 mM Tris-HCl (pH 8.5), 50 mM NaCl, 1 mM MgCl2, and 2 mM dithiothreitol, 0.1% Triton X-100, 0.22 mM of each dATP, dTTP, dCTP, dGTP, 0.28 μM of each primer, and 0.9 U of Taq polymerase (Promega, Madison, WI). The program consisted of one cycle of 3 minutes at 94°C, then 40 cycles of 20 seconds at 94°C, 20 seconds at 55°C, 40 seconds at 72°C, and finally one cycle of 3 minutes at 72°C. For the 1605 Air Thermo-Cycler (Idaho Technology), the PCR mixture contained 50 mM Tris-HCl (pH 8.5), 20 mM KCl, 3 mM MgCl2, 0.05% bovine serum albumin (BSA, No. A-4378, SIGMA, Chemical Co., St. Louis, MO), 0.25 mM of each dATP, dTTP, dCTP, dGTP, 0.25 μM of each primer, and 0.9 U of Taq polymerase. The program consisted of one cycle of 15 seconds at 94°C, then 30 cycles of (5 seconds at 94°C, 5 seconds at 55°C, 15 seconds at 74°C), and finally one cycle of 2 minutes at 74°C, 2 seconds at 45°C. The fastest transition speed (S-9 on the 1605 Air Thermo-Cycler and 2.0 on the Rapidcycler) was chosen.

For the MiniCycler, Perkin Elmer Cycler 480, and PHC-2, the PCR reaction has to be covered with 50 µl of mineral oil, but for the BioOven and the Air Thermo-Cycler, no oil was needed.

The PCR products (6 - 10 µl each) were separated by gel electrophoresis in a 2 - 3% agarose gel containing ethidium bromide (1 µg/ml).

Results and Discussion

Figure 1 shows the PCR results using the four different PCR cycler machines. Only the two Mycoplasma gallisepticum strains gave 138 bp PCR products (lanes 1 and 2). M. synoviae and H2O (lanes 3 and 4) were negative. The most intense bands were in panel d (Air Thermo-Cycler) and panel c (PHC-2) compared with less intense bands in panel a (MiniCycler) and panel b (BioOven).

Figure 2. PCR results for different bacterial species in the 1605 Air Thermo-Cycler. 105 cells of each bacterial species was used for this test. 2% agarose gel was used for the electrophoresis. Lane m: molecular size marker. Lane 1: Clostridium perfringens. The product is 280 bp. Lane 2: C. leptum. The product is 257 bp. Lane 3: C. clostridiiforme. The product is 255 bp. Lane 4: Bacteroides distanonis. The product is 273 bp. Lane5: B. thetaiotaomicron. The product is 423 bp. Lane 6: B. vulgatus. The product is 287 bp. Lane 7: Bifidoacterium sp. The product is 190 bp.

Cycle times were 50 seconds in Air Thermo-Cycler, 2 minutes 5 seconds in MiniCycler, 4 minutes in PHC-2, and 7 minutes 10 seconds in BioOven. The total cycle times were 30 minutes in 1605 Air Thermo-Cycler, 1.5 hours in the MiniCycler, 2.5 hours in the Perkin Elmer Cycler 480 (data not shown), 2.8 hours in the PHC-2, and 5 hours in the BioOven.

High concentrations of BSA were essential for the PCR assay in the 1605 Air Thermo-Cycler, BSA is thought to prevent denaturation of the Taq polymerase on the large internal surface area of the glass capillary tubes (6).

We have already used this protocol and the 1605 Air Thermo-Cycler to detect many other bacteria, such as Clostridium perfringens, C. Clostridiiforme, C. leptum, Bacteroides distasonis, B. thetaiotaomicron, B. vulgatus, and Bifidobacterium. Figure 2 shows the results. Different primers were used for different bacterial species, but the same program and same 1605 Air Thermo-Cycler

were used. All of them gave good results.

We directly used the bacterial cells lysed in 1% Triton X-100 for DNA template of the PCR, so the final concentration of the Triton X-100 in the reaction tubes was about 0.1%. This concentration of Triton X-100 did not interfere with the PCR assay (data not shown).

In general, the optimal annealing temperature used in the 1605 Air Thermo Cycler was 5°C lower than the other machines and gave better sensitivity and better specificity (Figure 3).

In conclusion, the 1605 Air Thermo-Cycler is the fastest and most sensitive PCR machine for the detection and identification of microbial pathogens.

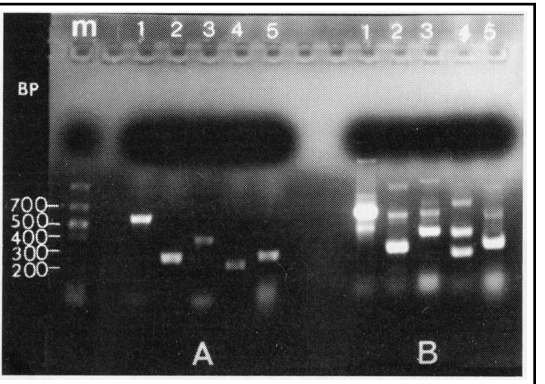

Figure 3. Comparison of the results of 5 PCR methods for 5 different bacterial species in the 1605 Air Thermo-Cycler (Idaho Technology) and the Cycler 480 (Perkin Elmer). Panel A: the 1605 Air Thermo-Cycler (25 μl tube, one cycle of 94°C for 15 sec, 30 cycles of 94°C for 3 sec, 50°C for 10 sec, 74°C for 15 sec and finally one cycle of 74°C for 2 min and 45°C for 2 sec). Panel B: the Cycler 480 (one cycle of 3 min at 95°C , then 35 cycles of 20 sec at 94°C, 20 sec at 55°C, 40 sec at 72 °C. and finally one cycle of 3 min at 72°C and 2 sec at 20°C). Line m, molecular marker. Lane 1: Escherichia coli, the primer set is CACACGCTGACGCTGACCA; with GACCTCGGTTTAGTTCACAGA, PCR product is 585 bp. Lane 2: Eubacterium limosum, the primer set is GGCTTGCTGGACAAATACTG; with CTAGGCTCGTCAGAAGGATG, the PCR product is 274 bp. Lane 3: Vibrio vulnificus, the primer set is CTCACTGGGGCAGTGGCT; with CCAGCCGTTAACCGAACCA, the PCR product is 383 bp. Lane 4: Listeria monocytogenes, the primer set is CGGAGGTTCCGCAAAAGATG; with CCTCCAGAGTGATCGATGTT, the PCR product is 234 bp. Lane 5: Staphylococcus aureus, the primer set is GCGATTGATGGTGATACGGTT; with CAAGCCTTGACGAACTAAAGC, the product is 276 bp.

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New From Idaho Technology

Kirk Ririe
Idaho Technology Inc.

Introducing the 1002 Rapidcycler

In March of 1995, we began shipping a new version of our capillary based temperature cycling system, the Rapidcycler. This system offers numerous advantages over the previous model.

Improved Temperature Control.

The Rapidcycler is able to run a broader range of temperature cycle protocols including two-temperature cycling. It is also less likely to overshoot elongation temperatures.

The temperature ramp rate between the annealing and elongation temperatures is now entered in degrees per second and is linear within and between runs.

Quiet Operation

The actuator used to control air flow through the Rapidcycler is a soft shift solenoid as opposed to the AC solenoid used in the 1605. This offers two advantages. Besides being much quieter, the new actuator allows intermediate door settings hence variable airflow. This is in contrast to the solenoid in the 1605 which had just two settings, open and closed. The new actuator allows the control software to more effectively dampen the temperature oscillations that tend to occur when driving rapid temperature changes.

Improved Programming

The Rapidcycler user interface is a significant improvement over the 1605. The readout size has been increased so that everything is printed in clear English instead of abbreviations. The three user modes, Cycle, Hold and Link, are now accessible by a single button from the keypad.

Maneuvering around programming screens has been simplified by the addition of cursor keys. There are now 99 Cycle programs, 99 Hold programs, and 99 Link programs available. Many of them come preprogrammed for the more commonly used reaction profiles.

New Optimizer Kit

The Optimizer kit has been modified to allow more flexibility and to reduce waste. There are now four base buffers available ranging in Mg++ concentration from 10 mM to 40 mM . Either of two gel loading additives (Ficoll/tartrazine or sucrose/cresol red) can be added along with dNTPs and other reaction constituents into a master mix which will keep for months in a refrigerator.

Blazer mini-torch

We now ship a Blazer butane torch with the Rapidcycler. The torch is fast igniting, light-weight, and burns very hot. It is a good general purpose lab torch and is ideal for sealing glass capillaries. We recommend that everyone using glass capillaries keep one handy. They are available either directly from us or from some sporting-goods outlets.

Research in Progress at IT

Idaho Technology Inc., together with the University of Utah has received generous funding from the National Institutes of Health STTR program and from the University of Utah. This joint research project is an effort to develop a system to continuously monitor the progress of an amplification reaction. The use of capillary tubes lends itself to fluorescent analysis of reaction product during the course of a reaction. By combining a fluorimeter and thermal cycler into a single mechanism it is possible to essentially "watch" a reaction occur. It is hoped that this research will lead to extremely rapid detection systems as well as becoming a general purpose window into reaction mechanics.

Index

C

Capillary tubes
- dispensing from 4-6, 15-20
- module 7, 18-20
- sealing 6, 16-20

Cycle Mode
- criteria 24
- editing programs 23-25
- parameters 23
- running a program 24-25
- table of programs 29-33

E

Editing Numbers 22
Electric fuse replacement 40

H

Hold Mode
- criteria 26
- editing programs 25-26
- parameters 25
- running a program 26-27
- table of programs 29-33

L

Light bulb replacement 38-39

Link Mode
- editing Programs 27
- parameters 27
- running a program 28
- table of programs 29-33

M

Microcentrifuge tubes
- oil overlays 13
- protocols 10
- reaction optimization 12-13
- real vs. set temperatures 13-14

P

Program Memory 28

S

Setting Up the RapidCycler 3

T

Thermal fuse replacement 40-41

Troubleshooting
- reaction problems 37
- slow cooling 35
- slow heating 36

W

Warranty information 46

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